PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/49, 15/86, A61K 39/21
C07K 13/00

A1

(11) International Publication Number: WO 92/22654

(43) International Publication Date: 23 December 1992 (23.12.92)

(21) International Application Number: PCT/US92/04980

(22) International Filing Date: 10 June 1992 (10.06.92)

(30) Priority data:
714,152 11 June 1991 (11.06.91) US

(71) Applicant: MICROGENESYS, INC. [US/US]; 1000 Research Parkway, Meriden, CT 06450 (US).

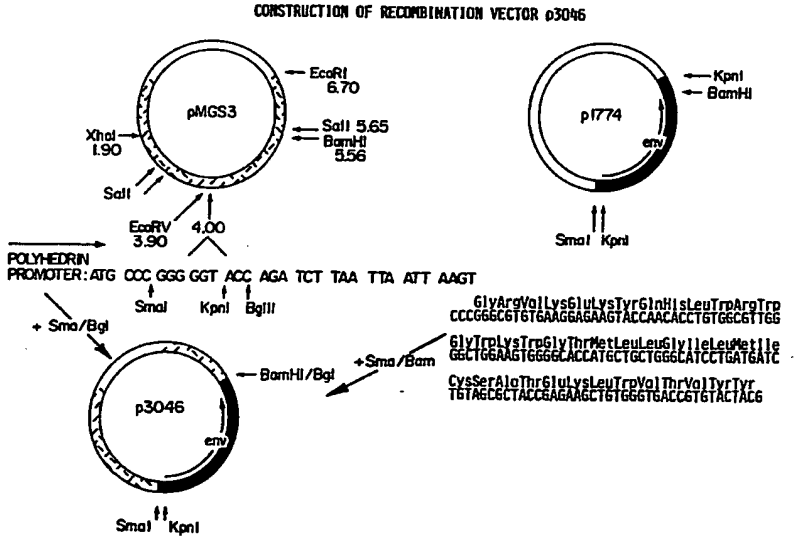
(72) Inventors: SMITH, Gale, E. ; 125 Mechael Drive, Guilford, CT 06606 (US). VOLVOVITZ, Franklin ; 123 York St. #18E, New Haven, CT 06511 (US).

(74) Agents: SCHAFFER, Robert et al.; Darby & Darby, 805 Third Avenue, New York, NY 10022-7513 (US).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL (European patent), NO, PL, RO, RU, SD, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: VACCINE AND TREATMENT METHOD OF HUMAN IMMUNODEFICIENCY VIRUS INFECTION



(57) Abstract

An Acquired Immunodeficiency Syndrome (AIDS) vaccine containing the Human Immunodeficiency Virus, Type-1 (HIV-1) envelope proteins is produced from cloned HIV-1 envelope genes in a baculovirus-insect cell vector system. The recombinant HIV-1 proteins are purified, assembled into particles and then adsorbed on an aluminum phosphate adjuvant. The resulting adsorbed recombinant HIV-1 virus envelope protein formulation (AIDS vaccine) is highly immunogenic in animals and elicits antibodies which bind to the HIV-1 virus envelope and neutralize the infectivity of the virus in *in vitro* tests. The above AIDS vaccine induces new humoral and cellular immune responses in HIV-infected patients and is useful as a form of vaccine therapy to delay or prevent the destruction of the immune system.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

VACCINE AND TREATMENT METHOD OF HUMAN IMMUNODEFICIENCY VIRUS INFECTION

This application is a Continuation-in-part of U.S. Patent Application Serial No. 151,976 filed February 3, 1988 which is a Continuation-in-part of U.S. Patent application Serial No. 920,197 filed October 16, 1986 (now Serial No. 585,266). These applications and the references cited herein are incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

The Human Immunodeficiency Virus Type-1 (HIV-1) is a retrovirus which causes a systemic infection with a major pathology in the immune system and is the etiological agent responsible for Acquired Immune Deficiency Syndrome (AIDS). Barre-Sinoussi, et al., Science, 220: 868-871 (1983); Popovic et al., Science, 224: 497-500 (1984). Clinical isolates of HIV-1 have also been referred to as Lymphadenopathy-Associated Virus (Feorino, et al., Science, 225: 69-72 (1984) and AIDS-related Virus (Levy et al., Science 225: 840-842 (1984)).

AIDS has become pandemic and the development of a vaccine has become a major priority for world public health. A high percentage of persons infected with HIV-1 show a progressive loss of immune function due to the depletion of T4 lymphocytes. These T4 cells, as well as certain nerve cells, have a molecule on their surface called CD4. HIV-1 recognizes the CD4 molecule through a receptor located on the envelope of the virus particles, enters these cells, and eventually replicates and kills the cell. An effective AIDS

vaccine might be expected to elicit antibodies which would bind to the envelope of HIV-1 and prevent it from infecting T4 lymphocytes or other susceptible cells.

Vaccines are generally given to healthy individuals before they are exposed to a disease organism as an immune prophylactic. However, it is also reasonable to consider using an effective AIDS vaccine in post-exposure immunization as immunotherapy against the disease. Salk, J., Nature, 327: 473-476 (1987).

It is widely believed that the HIV-1 envelope ("env") is the most promising candidate in the development of an AIDS vaccine. Francis and Petricciani. New Eng. J. Med., 1586-1559 (1985); Vogt and Hirsh, Reviews of Infectious Disease, 8: 991-1000 (1986); Fauci, Proc. Natl. Acad. Sci. USA, 83: 9278-9283. The HIV-1 envelope protein is initially synthesized as a 160,000 molecular weight glycoprotein (gp160). The gp160 precursor is then cleaved into a 120,000 molecular weight external glycoprotein (gp120) and a 41,000 molecular weight transmembrane glycoprotein (gp41). These envelope proteins are the major target antigens for antibodies in AIDS patients. Barin, et al., Science, 228: 1094-1096 (1985). The native HIV-1 gp120 has been shown to be immunogenic and capable of inducing neutralizing antibodies in rodents, goats, rhesus monkeys and chimpanzees. Robey, et al., Proc. Natl. Acad. Sci. USA 83:7023-7027 (1986).

Due to the very low levels of native HIV-1 envelope protein in infected cells and the risks associated with preparing an AIDS vaccine from HIV-1 infected cells, recombinant DNA methods have been employed to produce HIV-1 envelope antigens for use as AIDS vaccines. Recombinant DNA technology appears to present the best option for the production of an AIDS subunit vaccine because of the ability to produce large quantities of safe and economical immunogens. The HIV-1 envelope protein has been expressed in genetically altered vaccinia virus recombinants. Chakrabarti, et al., Nature, 320: 535-537 (1986); Hu, et al., Nature, 320: 537-540 (1986); Kieny, et al.,

Biotechnology, 4:790-795 (1986). The envelope protein has also been expressed in bacterial cells (Putney, et al., Science, 234: 1392-1395 (1986)), in mammalian cells (Lasky, et al., Science, 23:209-12 (1986)), and in insect cells. Synthetic peptides derived from amino acid sequences in an HIV-1 gp41 have also been considered as candidate AIDS vaccines. Kennedy, et al. (1986). However, a successful AIDS vaccine has not been produced using these materials and methods.

The use of a baculovirus-insect cell vector system to produce recombinant HIV-1 envelope proteins is one aspect of the invention disclosed in copending and coassigned U.S. patent application Serial No. 920,197 filed October 16, 1986 (now Serial No. 585,266). See also, Serial No. 151,976.

The baculovirus system has been demonstrated to be of general utility in producing HIV-1 proteins and other proteins. As examples, the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) has been used as a vector for the expression of the full length gp160 and various portions of the HIV-1 envelope gene in infected Spodoptera frugiperda (fall armyworm) cells (Sf9 cells). Also disclosed in the prior copending patent applications is the truncated gp160 gene (recombinant number Ac3046), the protein produced from recombinant Ac3046, and a purification technique for the Ac3046 gene product that includes lentil lectin affinity chromatography and gel filtration chromatography. The gp160 protein purified in this manner and aggregated to form particles was found to be highly immunogenic in rodent and primate species.

The ideal AIDS vaccine, in addition to the requirements of being substantially biologically pure and non-pyrogenic, should provide life-long protection against infection with HIV-1 after a single or a few injections. This is usually the case with live attenuated vaccines. When killed bacteria or viruses, or materials isolated from them, such as toxoids or proteins, are used to make a vaccine, there often results a poor antibody response and

only short term immunity. To overcome or minimize these deficiencies in a vaccine, an additional component, called an adjuvant, may be added. Adjuvants are materials which help stimulate the immune response. Adjuvants in common use in human vaccines are gels of aluminum salts (aluminum phosphate or aluminum hydroxide), usually referred to as alum adjuvants. Bomford, et al., "Adjuvants," Animal Cell Biotech. Vol. 2: 235-250, Academic Press Inc. (London: 1985).

The present invention provides a vaccine and treatment methods for human immunodeficiency virus (HIV), comprising the administration of recombinant HIV envelope protein to an infected or susceptible individual. In a preferred embodiment, the envelope protein may be purified, aggregated, and combined with an adjuvant (e.g., alum) for vaccine use.

BRIEF DESCRIPTION OF THE DRAWINGS

Details of this invention are set forth below with reference to the accompanying drawings:

Fig. 1 illustrates the cloning strategy used to isolate the HIV-1 envelope gene (env) from the E. coli plasmid pNA2. The hatched regions are HIV-1 DNA sequences and the open regions are from the cloning vectors. The black region in the plasmid p1774 is constructed from synthetic oligonucleotides and was introduced as an SmaI--KpnI fragment into the SmaI-KpnI sites of plasmid p1614. The sequence of this synthetic oligonucleotide is shown.

Fig. 2 illustrates the strategy used to construct the recombinant plasmid vector (p3046), which in turn is used to construct the baculovirus expression vector Ac3046. The plasmid pMGS3 contains sequences (cross-hatched areas) from the baculovirus AcNPV on either side of a cloning site at position 4.00. This site has the unique restriction endonuclease sites for SmaI, KpnI, and BglII. The AcNPV polyhedrin promoter is in the 5' direction from the 4.00 position. The sequence

5'-TAATTAATTAA-3' is in the 3' direction, and has a translational termination codon in all three reading frames. The plasmid p1774 and the sequence of the synthetic oligonucleotide region is as described in Fig. 1. The plasmid p3046 contains all of pMGS3 except for the sequences between the SmaI and BglII sites, where the HIV-1 envelope gene of p1774 is inserted.

Fig. 3 shows the nucleotide sequences of the DNA flanking the Ac3046 gp160 coding sequences. The 3046 env DNA sequence between +1 and +2264 is shown in Fig. 4.

Figs. 4a-4k show the actual DNA sequence of the HIV-1 env gene segment along with the synthetic oligonucleotide sequences at the 5' end of the env gene in Ac3046 (between +1 and +2264). The locations of restriction endonuclease sites are listed above the DNA sequence and the predicted amino acid sequence is listed below the DNA sequence. The bases are numbered on the right and on the left.

Figs. 5a-5d compare the DNA sequences of the env gene from Ac3046 with a published env gene sequence from LAV-1. The LAV-1 sequence is on the top and Ac3046 is on the bottom. A line (1) below the LAV-1 sequence indicates that the sequence in Ac3046 is the same in this position. The DNA sequence numbering used is that described by Wain-Hobson, et al., Cell, 40:9-17 (1985) for LAV-1.

Fig. 6 shows the ELISA end point dilution titers of human HIV-1 antibody positive sera (top graph) and rhesus monkey sera (bottom graph) from animals immunized with gp160 (IJ55, KL55) or gp120 (AB55, CD55, GH55). The ELISA titers were measured against highly purified gp120 and gp160 proteins. The specifically bound antibody was measured with a goat anti-human IgG HRP conjugate. The highest dilution of serum that gives a positive response in the test is the titer.

Fig. 7 is a Table summarizing the gp160 Vaccine-induced immune responses of vaccinated seropositive patients.

Fig. 8 (A and B) shows vaccine-induced antibody responses directed against specific HIV envelope epitopes.

Fig. 9 shows the vaccine-induced T-cell proliferative responses to gp160 in vaccinated seropositive individuals.

Fig. 10 (A-C) shows the lymphocyte proliferation responses associated with vaccination.

Fig. 11 is a graph showing the percent change in CD4 cells in responders and non-responders over time.

SUMMARY OF THE INVENTION

It has been discovered that recombinant HIV-1 gp160 envelope protein ("rgp160"), especially when adsorbed onto an adjuvant such as alum (e.g., aluminum phosphate) is particularly useful as an AIDS vaccine. One aspect of this invention is an AcNPV expression vector having the coding sequence for a portion of the HIV-1 envelope gene which encompasses the amino acids 1-757 found in the recombinant clone No. 3046. Another aspect of the invention is the production of that recombinant HIV-1 envelope protein (and the protein itself) in insect cells -- especially the rgp160 protein coded for by the amino acid sequences 1-757 (i.e., 03046).

Other aspects of this invention comprise purification and formation of recombinant envelope protein particles from the gene product of the recombinant baculovirus that produces the 3046 protein and adsorption of the 3046 particles to aggregates of aluminum phosphate.

The invention also comprises prophylactic and/or therapeutic vaccines for AIDS or HIV infection and methods of preventing or treating AIDS or HIV infection.

DETAILED DESCRIPTION OF THE INVENTION

The following examples illustrate the invention without limiting its scope.

The recombinant baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) which contains a truncated HIV-1 gp160 gene coding for amino acids 1-757 of

the HIV envelope protein (recombinant Ac3046) is described in copending, coassigned U.S. application Serial No. 920,197 (now Serial No. 585,260). The cloning steps employed to construct the recombinant baculovirus-containing genes or portions of genes from HIV-1 are also disclosed there and are incorporated by reference.

The following is a detailed description of the genetic engineering steps used to construct the Ac3046 expression vector. The materials employed, including enzymes and immunological reagents, were obtained from commercial sources. Examples showing how to make and use the invention are also provided.

Other recombinant envelope proteins, referred to collectively as rgp160, are also contemplated, and include recombinant gp120 and gp41 proteins. Ac3046 is just one example of an expression vector and recombinant envelope protein according to the invention.

EXAMPLE 1

Construction of the baculovirus recombinant Ac3046 bearing the HIV-1 coding sequences for amino acids 1-757

Cloning and expression of foreign protein coding sequences in a baculovirus vector requires that the coding sequence be aligned with the polyhedrin promoter and upstream sequences on one side and with baculovirus coding sequences on the other side. The alignment is such that homologous recombination with the baculovirus genome results in transfer of the foreign coding sequence aligned with the polyhedrin promoter and an inactive polyhedrin gene.

Accordingly, a variety of insertion vectors were designed for use in HIV envelope gene constructions. The insertion vector MGS3, described below, was designed to supply the ATG translational initiating codon. Insertion of foreign sequences into this vector must be engineered such that the translational frame established by the initiating codon is maintained correctly through the foreign sequences.

The insertion vector MGS3 was constructed from an EcoRI-I restriction fragment clone of DNA isolated from a

plaque purified AcMNPV isolate (WT-1). MGS3 was designed to consist of the following structural features: (a) 4000 bp of sequence upstream from the ATG initiating codon of the polyhedrin gene; (b) a polylinker introduced by site-directed mutagenesis, which consists of an ATG initiating codon at a position of the corresponding polyhedrin codon, and restriction sites SmaI, KpnI, BglII and a universal stop codon segment; (c) 1700 bp of sequence extending from the KpnI restriction site (which is internal to the polyhedrin gene) through to the terminal EcoRI restriction site of the EcoRI-I clone. See, e.g., Fig. 2.

EXAMPLE 2

Construction of baculovirus recombinants bearing LAV env coding sequences

A recombinant plasmid designated NA2 (Fig. 1) consists of a 21.8 kb segment of an entire HIV-1 provirus inserted into pUC18. This clone was reportedly infectious since it could produce virus following transfection of certain human cells. Adachi, et al., J. Virol. 59:284-291 (1986). The complete envelope gene sequences contained in NA2 were derived from the LAV strain of HIV. Barre-Sinoussi (1983).

The HIV-1 envelope gene was isolated and engineered as described below, and as shown in Fig. 1. The envelope gene was initially isolated from NA2 as a 3846 bp EcoRI/SacI restriction fragment and cloned into the EcoRI/SacI restriction site pUC19. The resultant plasmid was designated p708.

The envelope gene was subsequently reisolated as a 2800 bp KpnI restriction fragment and cloned into the KpnI restriction site of pUC18. The resulting clone was designated p1614.

The KpnI restriction fragment in p1614 contained a slightly truncated piece of the HIV envelope gene such that 121 bp of the N-terminal corresponding sequence was missing. This missing part in the gene, which included the signal peptide sequences, was replaced by insertion of a

double-stranded synthetic oligomer. The inserted oligomer was designed from the LAV amino acid sequence using preferred polyhedrin gene codon usage. To facilitate further manipulation, a new SmaI restriction sequence was concomitantly introduced in place of the ATG initiating codon. The ATG initiation codon will be supplied by the baculovirus insertion vector. The resultant plasmid was designated p1774.

Referring to Fig. 2, restriction fragments from p1774 containing coding sequences of various domains of the HIV-1 envelope were cloned into the MGS insertion vectors (e.g., MGS3) such that the ATG initiating codon of the insertion vector was in-frame with the codons of the envelope gene. Construct p3046 consisted of the SmaI/BamHI restriction fragment isolated from p1774 inserted into the SmaI/BglII site of the plasmid vector pMGS3. This clone contains sequences coding for amino acids 1 through 757 of gp160 and uses a termination codon supplied by the MGS3 vector.

EXAMPLE 3

Preparation and Selection of Recombinant Baculovirus

The HIV env gene recombination plasmid p3046 was calcium phosphate precipitated with AcMNPV DNA (WT-1) and added to uninfected Spodoptera frugiperda cells. The chimeric gene was then inserted into the AcMNPV genome by homologous recombination. Recombinant viruses were identified by an occlusion negative plaque morphology. Such plaques exhibit an identifiable cytopathic effect but no nuclear occlusions. Two additional successive plaque purifications were carried out to obtain pure recombinant virus. Recombinant viral DNA was analyzed for site-specific insertion of the HIV env sequences by comparing their restrictions and hybridization characteristics to wild-type viral DNA.

EXAMPLE 4

Expression of HIV env from recombinant

baculoviruses in infected insect cells

Expression of HIV env sequences from the recombinant viruses in insect cells should result in the synthesis of primary translational product. This primary product will consist of amino acids translated from the codons supplied by the recombination vector. The result is a protein containing all the amino acids coded for from the ATG initiating codon of the expression vector downstream from the polyhedrin promoter to the translational termination signal on the expression vector (e.g., rgp160). The primary translation product of Ac3046 should read Met-Pro-Gly-Arg-Val at the terminus where Arg (position 4) is the Arg at position 2 in the original LAV clone. The Met-Pro-Gly codons are supplied as a result of the cloning strategy.

EXAMPLE 5

Nucleotide sequence of the gp160 insert and flanking DNA.

The nucleotide sequence of the gp160 insert and flanking DNA was determined from restriction fragments isolated from viral expression vector Ac3046 DNA. The sequencing strategy involved the following steps. The 3.9 kb EcoRV-BamHI fragment was purified by restriction digestion of Ac3046 viral DNA. The Ac3046 viral DNA had been prepared from extracellular virus present in the media of cells being used for a production lot of vaccine.

As shown in Fig. 2, the 3.9 kb EcoRV-BamHI fragment consists of the entire gp160 gene and 100 bp of upstream and about 1000 bp of downstream flanking DNA. Of this, the nucleotide sequence of the entire gp160 gene was determined, including 100 bp of upstream and 100 bp of downstream flanking DNA.

Briefly, the results of the sequencing revealed a chimeric construct as predicted from the cloning strategy. The sequence of the gp160 was essentially as reported by Wain-Hobson, et al. (1985). The sequence of 2253 bases between the presumed translation initiation and termination

codons predicts 751 amino acid codons and 28 potential N-linked glycosylation sites. The estimated molecular weight of this rgp160, including the sugar residues, is approximately 145,000.

Sequence analysis of 200 bases of flanking DNA indicated correct insertion as shown in Figs. 3, 4 and 5.

EXAMPLE 6

Amino Acid Sequence of gp160

Using standard automated Edman degradation and HPLC procedures, the N-terminal sequence of the first 15 residues of gp160 was determined to be identical to that predicted from the DNA sequence. The N-terminal methionine is not present on the gp160 protein. This is consistent with the observation that AcNPV polyhedrin protein is also produced without an N-terminal methionine. A summary of the actual gp160 DNA and N-terminal protein sequences, as has been determined by analysis of the AcNPV 3046 DNA and purified gp160, is as follows (Table 1).

TABLE 1

LAV env gene in the AcNPV 3046 expression vector

Residue	2	3	4	5	6	7	8	9	10	11	12	13	14
	Pro	Gly	Arg	Val	Lys	Glu	Lys	Tyr	Gln	His	Leu	Trp	Arg	Trp Gly
	ATG	CCC	GGG	CGT	GTG	AAG	GAG	AAG	TAC	CAA	CAC	CTG	TGG	CGT TGG GGC

These results compare to the original LAV-1 clone as follows (Table 2).

TABLE 2

LAV env gene in the original LAV-1 clone

Residue	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Met	Arg	Val	Lys	Glu	Lys	Tyr	Gln	His	Leu	Trp	Arg	Trp	Gly
	ATG	AGA	GTG	AAG	GAG	AAG	TAT	CAG	CAC	TTG	TGG	AGA	TGG	GGG

EXAMPLE 7

Purification of Recombinant gp160

One aspect of the present invention is the procedure used to extract and purify the recombinant HIV-1 envelope protein coded for in the Ac3046 expression vector. The recombinant HIV-1 envelope protein gp160 is produced in S. frugiperda cells during 4-5 days after infection with Ac3046. Purification of this rgp160 protein involves the steps:

1. Washing the Cells
2. Cell Lysis
3. Gel Filtration Chromatography
4. Lentil Lectin Affinity Chromatography
5. Dialysis

This example describes the purification of the recombinant gp160 from about 2 x 10^9 Ac3046 infected cells.

1. Washing the cells. Infected cells are washed in a buffer containing 50 mM Tris buffer (pH 7.5), 1 mM EDTA and 1% Triton X-100. The cells are resuspended in this buffer, homogenized using standard methods, and centrifuged at 5000 rpm for 20 minutes. This process is repeated 3 times.

2. Cell Lysis. The washed cells are lysed by sonication in 50 mM Tris buffer (pH 8.0-8.5), 4% deoxycholate and 1% beta mercaptoethanol. Sonication is done using standard methods. After sonication, only remnants of the nuclear membrane are intact and these are removed by centrifugation at 5000 rpm for 30 minutes. The supernatant containing the extracted gp160 has no intact cells, as determined by light microscopy observations.

3. Gel filtration. Gel filtration is done in a Pharmacia 5.0 x 50 cm glass column packed with a Sephacryl resin (Pharmacia). The total bed volume is about 1750 ml. To depyrogenate and sanitize the column and tubing connections, at least 6 liters of 0.1 N NaOH is run through the column over a period of 24 hours. The effluent from the

column is connected to a UV flow cell and monitor and a chart recorder (Pharmacia) and then is equilibrated with 4 liters of Gel Filtration Buffer. The crude gp160 is loaded onto the column and is developed with Gel Filtration Buffer.

The column separates the crude mixture into three major UV absorbing fractions. The first peak comes off between about 500 and 700 ml, the second between 700 and 1400 ml and the third between 1400 and 1900 ml buffer. This same profile is observed on small analytical columns from which it has been determined that the first peak is material that has a molecular weight of $\geq$ 2,000,000.

This peak is translucent due to a concentration of high molecular weight lipids and lipid complexes. This peak also contains from 10% to 20% of the gp160 extracted from the infected cells. Apparently this fraction of gp160 is complexed to itself or other cell components to form high molecular weight aggregates.

The second broad peak contains the majority of the gp160 and proteins with molecular weights of between about 18,000 and 200,000.

The third peak contains little protein and the majority of the UV absorption is due to the beta mercapto-ethanol in the sample.

When the second peak is first detected from the tracing of the UV absorbance, the effluent from the column is applied directly onto the lentil lectin column. Once the second peak has come off the column, the effluent is disconnected from the lentil lectin column and directed to waste.

4. Lentil Lectin. The lentil lectin affinity gel media (Lentil Lectin-Sepharose 4B) was purchased in bulk from Pharmacia. The lentil lectin was isolated by affinity chromatography on Sephadex to greater than 98% purity and then was immobilized by coupling to Sepharose 4B using cyanogen bromide. The matrix contains about 2 mg ligand per ml of gel. The lentil lectin column is a 5.0 x 30 cm glass column (Pharmacia) containing 125 ml lentil lectin-Sepharose

4B gel. The affinity matrix is reused after being thoroughly washed and regenerated by a procedure recommended by the supplier. When not in use, the gel is stored in the column in a solution of 0.9% NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.01% thimerosal. The column is washed and equilibrated with 250 ml lentil lectin buffer described above before each use.

The crude gp160 is applied to the column directly as it is eluting from the gel filtration column as described above. Once the crude gp160 is bound to the column, it is washed with 800 ml lentil lectin buffer containing 0.1% deoxycholate. Under these conditions all of the gp160 binds to the column. Lentil lectin buffer plus 0.3M alpha-methyl mannoside is used to elute the bound glycoproteins which is monitored through a UV monitor at a wavelength of 280 nm.

5. Dialysis. Sugars and deoxycholates are removed by conventional dialysis.

The purification of gp160 from 1 liter of infected cells can be summarized in the following table (Table 3).

In another embodiment, conventional ion exchange chromatography (anionic or cationic) may be used in place of gel filtration. Similarly, the order of steps is not critical: For example, gel filtration or ion exchange chromatography may follow the lentil lectin purification step. Other reagents may also be used according to the invention. For example, other detergents may be used to purify the recombinant protein in place of deoxycholate. These include nonionic detergents such as Tween 20 (polysorbate 20), Tween 80, Lubrol, and Triton X-100.

TABLE 3 - Purification Summary

Purification Step	Total Protein (mg)[1]	gp160 Protein (mg)	%gp160 Total	Contaminants Removed
Cell Pellet	1-2000	20	1-2	Culture Medium
1,2,3rd Wash	250	15	6	Serum Albumin, most Nucleic Acids, and Soluble Cell Proteins
Gel Filtration	120	12	12	Lipids, Nucleic Acids, and high mol wt aggregates
Lentil Lectin	14	10	70	Nonglycosylated proteins
Dialysis	13	9	70	Sugar, deoxycholate, excess Tris buffer

EXAMPLE 8

A. Assembly of gp160 Particles.

As one aspect of the present invention, it has been discovered that the gp160 antigen can be assembled into particles of $\geq$ 2,000,000 molecular weight during purification. The gp160 protein is extracted from the cell as a mixture of 80-90% monomeric (160,000 molecular weight) and 10-20% polymeric (particle form). The gel filtration step removes the aggregated forms of gp160. Attempts to purify the gp160 from this fraction (first peak off the gel filtration column) suggest that it is complexed with other cell proteins, possibly even with membrane fragments. However, the gp160 antigen in the second peak off the gel filtration column has a molecular weight of about 160,000-300,000 and is, therefore, in predominantly monomeric or dimeric form.

The formation of aggregates or polymers of gp160 occurs during the development of the lentil lectin column. It has been determined that the antigen forms aggregates whether it is eluted from the lectin column in 0.5% deoxycholate, which is about the 0.2% critical micelle con-

[1] Total protein was estimated from absorbance at 280nm.

centration (CMC) for deoxycholate, or whether the gp160 is eluted from the column in 0.1% deoxycholate.

The size of the aggregates are measured on a high resolution FPLC Superose 12 column (Pharmacia). Samples from representative lots of purified gp160 have a size that is predominantly equal to or greater than the 2,000,000 molecular weight of a blue dextran size standard.

A cross-linking study by Schwaller, et al. (1989), demonstrated that gp160 produced in insect cells is a tetramer of identical submits. The study also shows that gp160 in HIV-infected cells and virus particles is tetrameric. Thus, the recombinant gp160 particles may have tertiary and quaternary structures that are similar to those found in the native HIV gp160.

Proper 3-dimensional structure could be important for the formation of epitopes that require correct folding of gp160. It is likely that, as non-glycosylated proteins are removed from association with the gp160 antigen during the binding and washing to the lentil lectin column, the hydrophobic portions of gp160 begin to form intermolecular associations. The deoxycholate is probably not bound to the gp160 as the concentration can be kept above the CMC and the antigen will still form complexes. The assembly of this antigen into aggregates appears to be an intrinsic property of this protein once it is purified according to the invention. It is possible that the very hydrophobic N-terminal sequence that is present on the gp160 protein contributes to the natural ability of this protein to form particles. After purification, the gp160 complexes can be sterile filtered through a 0.2 micron cellulose acetate filter without significant loss of protein.

B. Analysis of Particle Formation.

An analysis of purified gp160 particles by electron microscopy demonstrates that they are protein-like, spherical particles of 30-100 nM.

As an additional test for the presence of particles, purified gp160 was analyzed by gel filtration.

About 100 micrograms of gp160 was applied to a Superose 12, FPLC gel filtration HR 10/30 column (Pharmacia, Inc.). This column was first calibrated with protein molecular weight standards. The protein profile from this column is highly reproducible; the elution volume is inversely proportional to the molecular weight of the protein standards. The column separates the monomeric gp160 from the polymeric forms and excludes globular proteins of $\geq 2 \times 10^6$ molecular weight. When developed on this column, essentially all of the purified gp160 elutes in the void volume and is, therefore, $\geq 2 \times 10^6$ (2,000,000) molecular weight in size.

EXAMPLE 9

A. Adsorption of gp160 to Alum.

The effectiveness of insoluble aluminum compounds as immunologic adjuvants depends on the completeness of adsorption of the antigens on the solid phase. As part of the present invention it was discovered that alum compositions could be made that would efficiently adsorb the gp160 but at a pH that would not reduce the potency of the gp160-alum complex as an immunogen. The factors controlled during the formation of this alum (aluminum phosphate gel) composition are:

1. The optimal pH for adsorption of antigens to alum is about 5.0. However, it was discovered that the gp160 lost immunogenicity at a pH of 6.5 in comparison to a pH of 7.5 so the alum is made at a pH of 7.1 ± 0.1. It was discovered that essentially 100% of the gp160 will still adsorb to the alum at this pH.

2. The ionic strength from the NaCl present is relatively low and is less than 0.15 M.

3. There is a molar excess of aluminum chloride relative to sodium phosphate to assure that there is

an absence of free phosphate ions in the supernatant.

4. The gp160 antigen is added to freshly formed alum to stop crystal growth and minimize the size of the particles.

The procedure to make 200 ml alum and adsorb purified gp160 to the alum is such that the final concentration of antigen is 40 μg/ml, as outlined below.

B. Preparation of Reagents (200 ml total formulated lot).

Prepare the following solutions in 100 ml sterile, pyrogen-free bottles or beakers. Mix the salts for Solution 1 and Solution 2 and the sodium hydroxide and filter through 0.2 micron cellulose acetate filters into 100 ml sterile, pyrogen-free bottles.

Solution 1	$AlCl_3.6H_2O$	0.895 grams
	$NaHAc.3H_2O$	0.136 grams
	Dissolve in 40 ml water for injection (WFI), 0.2 micron filter	
Solution 2	$Na_3PO.12H_2O$	1.234 grams
	Dissolve in 40 ml WFI, 0.2 micron filter	
Solution 3	NaOH	2.0 grams
	Dissolve in 100 ml WFI, 0.2 micron filter	
Solution 4	Tris	1.25 grams
	Dissolve in 100 ml WFI, add 1 ml to 90 ml WFI, adjust pH to 7.5 with 0.5N HCl, and bring to 100 ml with WFI	

Autoclave the solutions for 30 min; slow exhaust. Cool to room temperature.

C. Formation of Alum

1. Add Solution 1 (aluminum chloride-sodium acetate to the formulation vessel using 25 ml sterile,

disposable pipets. Note the volume of Solution 1 and begin stirring the solution.

2. Add Solution 2 (sodium phosphate) to the vessel using 25 ml sterile, disposable pipets and continue stirring as the precipitate forms and note the volume of Solution 2.

3. Add 3 ml Solution 3 (sodium hydroxide) and continue stirring for 5 min. Take a 0.5 ml sample and measure the pH. If the pH is less than 7.0, add an additional 0.5 ml sodium hydroxide, stir for another 5 minutes and measure the pH again. Continue until the pH is between 7.0 and 7.2.

4. Determine the total volume added to the formulation vessel (Solution 1 + Solution 2 + Solution 3), then add sterile WFI to bring the volume to 100 ml.

5. Immediately add 8,000 micrograms of purified gp160 in 100 ml of 1 mM Tris pH 7.5 directly into the formulation vessel.

6. Continue stirring for a minimum of 20 minutes, then dispense the formulated vaccine into sterile vials.

EXAMPLE 10

Immunogenicity of Alum Absorbed gp160 (Specific Ab Response)

An accepted method to determine the immunogenicity of an antigen preparation (vaccine) is to measure the specific antibody response in groups of mice which have been given a single dose of antigen. At the end of 4 weeks the mice are bled and the serum antibody levels to a specified antigen (usually the antigen used to immunize the animal) are measured by a standard antibody test, e.g. an ELISA (enzyme linked immunosorbent assay).

The immunogenicity in mice of purified gp160 with no adjuvant at pH 6.0 and pH 7.5 adsorbed with alum (as described in Example 9) or mixed with Freund's Complete Adjuvant are summarized below (Table 4).

TABLE 4

Group			gp160 Mean ELISA	Seroconversion	
gp160	Adjuvant	Lot#	OD[2]	%	(P/N)[3]
1 μg	None, pH 7,5	8702	0.140	57%	4/6
	None, pH 6.0	8702	0.110	26%	2/7
	Alum	8702	1.000	90%	9/10
	Alum	8705	2.285	100%	6/6
	Freund's	8604	1.108	83%	5/6
	Freund's	8702	1.396	100%	7/7
0.1 μg	Freund's	8604	0.434	67%	4/6
	Alum	8705	1.003	67%	4/6

Mice immunized with a single 1.0 microgram dose of gp160 antigen without any added adjuvant will elicit an antibody response against gp160 (see table above). However, a much stronger antibody response is seen in groups of mice immunized with 1.0 microgram of gp160 adsorbed to the alum adjuvant. A single dose of less than 0.1 microgram of gp160 mixed with complete Freund's or formulated with alum will seroconvert ≥ 50% of the immunized mice. Although less so, the gp160 antigen was immunogenic in mice as an unformulated antigen at pH 7.5 and at pH 6.0, but there was a loss of immunogenicity at the lower pH.

---

[2] The mice were bled 28 days post immunization and the sera tested at 1:10 dilution in an ELISA assay against gel-purified gp160. Similar results were obtained using a commercial ELISA (Genetic Systems Inc.; EIA™ ELISA) assay against the native HIV-1 proteins at a serum dilution of 1:400.

[3] The number of seroconverted mice (P) to the total number tested (N).

EXAMPLE 11

Immunogenicity of Alum Absorbed gp160 (ELISA Serum Study)

The ability of a candidate vaccine to elicit an immune response is a very important biological property. To confirm that the alum formulated gp160 vaccine was immunogenic in animals and to confirm that the alum adjuvant increased this immunogenicity, the following experiment was performed.

On day 0, mice (groups of 10) were injected with a single dose (0.5 micrograms, 1.0 micrograms, or 5.0 micrograms) of gp160 alone, gp160 adsorbed to alum or gp160 in complete Freund's adjuvant (CFA). On day 28 the mice were bled and the sera examined by ELISA (1:10 dilution) for the presence of antibodies to gp160.

Results from the sera drawn on day 28 are summarized in the table below (Table 5). In all groups, greater than 50% of the mice showed seroconversion. At all doses the number of sero-conversions and the average serum absorbance (OD_{450} nm at a 1:10 dilution in the ELISA assay) were higher with gp160 adsorbed to alum than those obtained in mice immunized with gp160 alone.

These results demonstrate that the alum adjuvant significantly increased the immunogenicity of the gp160 antigen.

TABLE 5 - 28 Days Post-Injection

	0.5 μg Dose		1.0 μg Dose		5.0 μg Dose	
	Mean		Mean		Mean	
	P/N[4]	OD[5]	P/N	OD	P/N	OD
gp160	9/10	.407	7/10	.699	7/10	.430
gp160 (alum)	9/10	.547	8/10	.797	10/10	1.347
gp160 (CFA)	10/10	1.130	10/10	1.967	10/10	1.317

EXAMPLE 12

Neutralization Data

HIV-1 neutralization assays are an accepted method to determine whether an antibody preparation will inhibit the HIV-1 virus from infecting susceptible human cultured lymphocyte cells. Antisera from animals immunized with gp160 were tested in an HIV-1 neutralization assay and the results are summarized in the table below (Table 6).

[4] The number of mice that seroconverted (P) compared to total number tested (N) at 28 days after being immunized with 0.5 micrograms, 1 micrograms or 5 micrograms of VaxSyn™ HIV-1.

[5] The mean absorbance (OD_{450}) of the mice that seroconverted as measured by the sponsor's ELISA assay against gp160 at a 1:10 dilution of serum.

TABLE 6

Animal	Identification	Immunogen/ Adjuvant	Micrograms[6]	Neutralizing Titer[7]
Rhesus	G55	gp120/Alum	16/8/8	1:80-1:160
Rhesus	H55	gp120/Alum	16/8/8	1:80-1:160
Rhesus	L55	gp160/Alum	16/8/8	≥ 1:80
Mice	Pool 3	gp120/Freund's	.25/.25/.25	1:40-1:80
Mice	Pool 8	gp160/Freund's	.1/.1/.1	1:40-1:80
G. Pig	Purified IgG	gp160/Freund's	10/10/10	1:320

Guinea pigs, rabbits and rhesus monkeys have also been immunized with gp160 (using alum or Freund's as an adjuvant). In general, the immunization of these animals has produced a good antibody response against the HIV-1 envelope proteins.

EXAMPLE 13

Immunogenicity in Chimpanzees

Genetically, the chimpanzee is man's closest relative and is currently the only animal model for infection of HIV-1. In a safety/immunogenicity trial in three chimpanzees, two chimpanzees were immunized with 40 micrograms or 80 micrograms of gp160 in an alum formulated vaccine. Each received a booster immunization at 4 weeks with 40 micrograms and 80 micrograms of gp160, respectively. A control animal was vaccinated at the same time with a 1 ml saline solution. Weekly serum samples were analyzed from each of the three chimpanzees for antibodies to gp160 and to HIV-1 viral antigens using three immunological assays, an ELISA assay against purified gp160 developed by

[6] Micrograms of gp160 or gp120 administered during the first/second/third immunization.

[7] The highest dilution of antisera that will inhibit the infection by 50% relative to HIV-1 infected cells that were exposed to serum from non-immunized animals.

MicroGeneSys, Inc., Western Blot analysis, and a commercial HIV-1 ELISA assay. The results of these analyses are described below.

A. ELISA (MGSearch HIV 160)

The ELISA assay, MGSearch HIV 160, MGSearch being a trademark of MicroGeneSys, Inc. of Meriden, Connecticut, U.S.A., is an immunosorbent assay against gp160 and is described in copending coassigned U.S. patent Application Serial No. 920,197 (now No. 585,266).

Serum samples taken before immunization and for the 11 weeks following the primary immunization were diluted from 1:10 to 1:100,000 and then incubated with nitrocellulose strips containing a 100 μg purified gp160 in a spot. The end point dilution titer is the highest dilution in which the test was positive for anti-gp160 antibody as detected with a goat anti-human IgG-alkaline phosphatase conjugate.

The serum samples from the control animal and from the pre-immune sera of the immunized animal were negative. The chimp which received the 80 microgram dose was positive at a 1:100 dilution by week 2 and the chimp which received a 40 microgram dose was positive at a 1:10 dilution by week 4. The antibody titers to gp160 continued to increase until week 5, at which time the end point dilution titers were approximately 1:100,000 and 1:2,000,000 respectively. The antibody titer in both animals dropped just slightly during weeks 6-11.

This type of response is similar both quantitatively and qualitatively to antibody responses commonly observed in chimps that have been vaccinated with a human Hepatitis B Virus vaccine.

B. Commercial ELISA Test

It was clear from the MGSearch HIV 160 ELISA and Western blot analyses of sera from the VaxSyn[8] immunized

[8] VaxSyn is a trademark of MicroGeneSys, Inc. for the AIDS vaccine described herein.

chimpanzees, that they had seroconverted and have antibodies against the recombinant gp160. To determine if they were also making anti-HIV antibody which recognized the native viral envelope proteins, the pre-immune sera and sera from weeks 1 through 11 were tested in a licensed, commercial ELISA test kit, the LAV EIA™ test kit of Genetic System Corporation, Seattle, Washington. The animal immunized with 80 micrograms of gp160 was positive at a 1:100 dilution by week 2 and continued to show an increase in antibody level through week 6. The animal immunized with 40 micrograms was positive at a 1:100 dilution by week 6.

EXAMPLE 14

Distribution of Antibodies Between gp120 and gp41

It is important to determine whether the antibody responses against gp160 in a vaccinated animal is directed against gp41, gp120 or both. A variety of immunological methods, including radioimmunoprecipitation (RIP), immunofluorescence (IF), Western blot analysis (WB), and quantitative ELISA against three different recombinant envelope antigens were employed to detect and measure for the distribution of antibodies against various regions of the HIV-1 envelope proteins.

Fig. 6 summarizes the immunoreactivity of three different recombinant antigens: [ART] [TAB] (1) gp120-delta (truncated recombinant HIV-1 gp120 with about 40 amino acids missing from the C-terminus of the molecule); [ART] [TAB] (2) gp120 (full length recombinant HIV-1 gp120; and [ART] [TAB] (3) gp160.

Human sera from 50 HIV-1 antibody positive individuals and 3 pooled human sera were highly reactive with gp160, moderately reactive with gp120 and little or no antibody reacted with truncated gp120. It is likely that the truncated gp120, which represents more than 90% of the HIV-1 external glycoprotein, contains protective determinants. The observation that human AIDS positive sera have few antibodies to this region of the envelope is consistent with the fact that the immune response to viral infection is

not fully protective and that human positive sera usually exhibit a low-level of neutralizing activity in vitro.

In contrast, rhesus monkeys immunized with either the gp160 immunogen or with the truncated gp120 have antibodies that react strongly with the truncated gp120 portion of the HIV-1 envelope. This difference in distribution of antibody recognition sites along the viral envelope and the higher titers observed in the monkeys may account for the fact that the monkey sera had high neutralizing titers.

A quantitative assessment of the immunoreactivity of these three recombinant envelope antigens with human and immune rhesus sera is presented in Fig. 7. All the monkey sera tested had high titer antibody against the truncated gp120 antigen (gp120-delta), including those from animals immunized with gp160.

These results demonstrate that the recombinant gp160 elicits an antibody response in rhesus monkeys that is different than what often occurs during natural infection. There are epitopes in the gp120-delta region of gp-160 that are efficiently recognized in the immunized monkeys that are not seen by the human immune system during infection. These new epitopes may be important for protection against HIV-1, and could be an important property of the recombinant gp160 for prevention and treatment of HIV-infection.

EXAMPLE 15

Therapeutic Vaccine Administration

A clinical trial with 30 HIV-seropositive human patients was conducted to determine the effects of vaccination with cloned HIV gp160 (produced in the baculovirus system as described above) on HIV infected individuals.

Vaccination with the recombinant gp160 led to an augmentation in the gp160 HIV-specific humoral and cellular immune responses of 19 out of 30 (63%) HIV seropositive volunteers. Fourteen out of 15 (93%) volunteers receiving 6 doses of the vaccine demonstrated an increase in their total gp160 antibody. Therefore, recombinant HIV proteins

(i.e., rgp41, rgp120, rgp160 and admixtures thereof) can be advantageously administered in a method to treat a human patient infected by HIV.

The effective amounts of HIV protein used in this embodiment of the invention can be determined according to techniques well known in the art, such as those presented below. In general such effective amounts may range between about 1 microgram and about 100 micrograms per kilogram body weight of the patient. The frequency of administration can also be determined by known means. In a preferred embodiment, administration is via the parenteral route, i.e., intravenously, intraperitoneally, intramuscularly, intradermally, etc., as is well known by those of ordinary skill in the art.

A. Volunteer Selection

Thirty volunteers with HIV infection were recruited. Only seropositive volunteers with early stage HIV infection, defined as Walter Reed Stage 1 or 2 (CD4 cell count not less than 400 for greater than 3 months, with or without lymphadenopathy) were eligible for enrollment. (Redfield, et al., New Engl. J. Med. 314: 131-132 (1986). Additional entry criteria limited volunteers to adults between the ages of 18 and 50, with a normal complete blood count, no evidence of end organ disease, no alcohol or drug abuse over the preceding 12 months, and who were not receiving anti-retroviral or immunomodulatory drugs. All patients underwent a 2 month baseline evaluation prior to randomization into treatment groups. No volunteers received any antiretroviral or immunomodulatory drugs during the trial.

Twenty-six of the 30 volunteers were men; 4 were women. Fourteen were Caucasian, 13 Black, and 3 Hispanic. The mean age was 29 (range 18-49). At enrollment 8 volunteers were Walter Reed Stage 1 and 22 volunteers were Walter Reed Stage 2. The baseline mean CD4 count was 668 (range 388-1639). The mean time between initial diagnosis and study entry was 24 months (range 3 months to 49 months).

B. Vaccine Product and Immunization Schedule

As described herein, the test vaccine comprises a non-infectious subunit glycoprotein derived from gp160 as a baculovirus expressed recombinant protein. The immunogenic protein was produced in Lepidopteran insect cells, was biochemically purified, and was adsorbed to aluminum phosphate for final vaccine formulation.

Three dose formulations of gp160 were used: 40 micrograms per milliliter, 160 micrograms per milliliter and 320 micrograms per milliliter. The injection volume for both the 40 μg and 160 μg dosages was 1 ml; 2 ml of 320 μg per milliliter was used to deliver the 640 μg dose injections.

The thirty volunteers were distributed into six groups of five volunteers each. Two immunization schedules were investigated: Schedule A, with vaccination on days 0, 30, and 120; and Schedule B, with vaccination on days 0, 30, 60, 120, 150 and 180. Within each immunization Schedule (A or B) there were three groups which received different dosages of vaccine (Table 7 below). All vaccinations were administered by intramuscular injection into the deltoid muscle. The duration of the trial was 10 months: a 2 month baseline evaluation, and an 8 month follow-up evaluation after the initial vaccination.

TABLE 7 - Immunization Schedule

	Amount of gp160 Administered (μg)					
Day	0	30	60	120	150	180
Schedule A						
Group 1	40	40		40		
Group 3	160	160		160		
Group 5	640	640		640		
Schedule B						
Group 2	40	40	40	160	160	160
Group 4	160	160	160	640	640	640
Group 6	640	640	640	640	640	640

C. Assessment of Safety and Toxicity

Each volunteer was interviewed and examined on days 0, 1, 2, 3, 15 and 30 after each injection. Volunteers were queried concerning fever, chills, nausea, vomiting, arthralgia (painful joints), myalgia (muscular pain), malaise, urticaria (hives), wheezing, dizziness, or headache. Examinations to assess local reactions at the site of injection included erythema, swelling, itching, pain and tenderness, skin discoloration, skin breakdown, change in regional lymphadenopathy, change in function of the injected extremity, and subcutaneous nodule formation at the site of injection. Monthly complete blood counts, serum chemistries, coagulation profile and urine analysis were also assessed.

In vitro cellular immune function was assessed by T-cell phenotyping (total lymphocyte, CD4 and CD8 cell phenotypes) as described in Rickman, et al., Clinical Immuno. 52: 85-95, 1989; Birx, et al., J. Acquir. Immune Defic. Syndr. 4: 188-196, 1991). T-cell proliferative response to mitogens (pokeweed and Con A) and control antigens (Candida albicans and tetanus) was also evaluated. Birx et al, supra. In vivo cellular immune function was assessed by delayed hypersensitivity skin testing to control antigens (i.e., mumps, tetanus toxoid, Candida albicans and trichophyton).

Quantitative viral cultures of peripheral blood mononuclear cells (PBMC) and plasma were assessed as described in Burke, et al., J. Acquir. Immune Defic. Syndr. 3: 1159-1167, 1991. DNA polymerase chain reaction (Wages, et al., J. Med. Virol. 33: 58-63, 1991) and serum p24 antigen levels were assessed to monitor in vivo HIV viral load.

No evidence of systemic toxicity was observed, but local reactogenicity was noted in 87 percent of the subjects (13 in each vaccination group). Local reactions included induration, tenderness, and transient subcutaneous nodule formation at the injection site; an increase in regional adenopathy was rarely noted. No subject refused a booster

injection. No difference in the frequency of local reactions was observed for primary immunization, booster injection, or dosage.

No evidence of adverse effects on the immune system was demonstrated as measured in vitro by mitogen and antigen specific proliferative responses, in vivo by delayed hypersensitivity skin testing responses, or by acceleration of quantitative CD4 cell depletion. Baseline mean CD4 cell counts were 716 and 605 for vaccine responders and non-responders, respectively. Mean CD4 cell counts from study days 180-240 were 714 and 561, for vaccine responders and non-responders, respectively. During the course of the 240-day trial, the net change in mean CD4 cell counts for vaccine responders was a minus 0.2 percent, while among vaccine non-responders the mean CD4 cell count declined by 7.3 percent (Figure 11). Vaccine induced HIV immunogenicity was not associated with evidence of accelerated CD4 decline in any individual subject throughout the entire course of the trial.

To assess the possibility of increased HIV replication and viral load in subjects as a consequence of vaccination, in vivo viral activity was measured by quantitative plasma and PBMC viral cultures, PBMC DNA polymerase chain reaction, and serum levels of p24 antigen. Quantitative cultures and DNA polymerase chain reaction assays demonstrated no alteration during this trial. Serum p24 antigen was undetectable in the subjects.

D. Assessment of Immunogenicity

Antibodies directed against whole HIV proteins were measured using both recombinant produced viral gene products gp160, p66, p24 and whole viral lysate of prototype HIV strain MN. Dot blot and Western Blot techniques were used, as described in Toubin, et al., Proc. Natl. Acad. Sci. USA 76: 4350-4354 (1979). Antibody responses to specific envelope epitopes were also measured (see Fig. 7).

In Fig. 7 epitopes 88 (amino acids 88-98 in gp120) and 448C (amino acids 448-514 in gp120) were selected

because antibody directed against these regions of gp120 are reported to correlate with early stage HIV infection.

Epitopes 106 (amino acids 106-121 in gp120), 241 (amino acids 241-272), 254 (amino acids 254-272), 300 (amino acids 300-340), 308 (amino acids 308-322), 422 (amino acids 422-454) and 735 (amino acids 735-752) were selected because of their putative functional importance. Epitopes 106 and 422 have been implicated in CD4 binding; epitopes 241, 254 and 735 have been implicated in group specific neutralization; and epitopes 300 and 308 have been implicated in type-specific neutralization).

Epitope 582 (amino acids 582-602) was selected as a control because it represents the immunodominant envelope domain in natural HIV infection. Additional epitopes investigated included 49 (amino acids 49-128); and 342 (amino acids 342-405).

In Fig. 7, a shaded box signifies a documented change in the HIV envelope-directed immune response. Shaded boxes with (=) signify a primary humoral response; shaded boxes with (+) signify a secondary humoral response; (-) signifies antibody negative to specific epitope pre and post immunization; and a (+) signifies antibody positive to specific epitope pre and post immunization, but without a quantitative change. Shaded boxes with (.) signify new T-cell proliferative response to gp160 following immunization. A (.) alone signifies no cellular response to gp160; while hb signifies "high background" (not interpretable); and nd signifies "not done."

Neutralization activity was measured against three prototype isolates (HIV-IIIB, RF and MN) in a syncytium inhibition assay as described in Nara, Nature, 333:469-470 (1988). HIV specific cellular responses were measured by known lymphocyte proliferation assay techniques using gp160, p24 and baculoviral expression system control protein (Birx, supra).

E. Vaccine Responders and Non-Responders

Subjects were classified as vaccine responders only if a reproducible selective increase of both a cellular and humoral immune response against HIV envelope specific epitopes were associated with the vaccination series (Fig. 7). Vaccine induced humoral immunity was defined as seroconversion to HIV envelope specific epitopes and/or a secondary booster immune response to envelope specific epitopes. Vaccine induced cellular immunity was defined as the development of a new, reproducible, vaccine associated, proliferative response to gp160.[9] Subjects who developed neither a humoral nor a cellular proliferative response or who developed only a humoral or only a cellular proliferative response to gp160 epitopes or HIV envelope were classified as non-responders.

F. Vaccine Induced Humoral Responses

Referring to Fig. 7, 19 of the 30 subjects (63 percent) demonstrated a vaccine induced augmentation of both gp160 HIV specific humoral and a cellular immune responses. These 19 were classified as "vaccine responders". Four of the 11 "non-responders" developed only a humoral or a cellular immune response. All 7 subjects who failed to demonstrate any detectable vaccine induced response received only 3 doses (Schedule A). No changes in antibody binding to HIV polymerase (p66), or structural (p24) gene products or the non-HIV control antigen tetanus were detected. No anti-baculoviral Lepidopteran cell control protein antibody developed in any subject.

Increases in envelope antibody (gp160) were detected in 13 subjects by Western Blot using the whole virus lysate HIV-MN. The changes were related to the immunization schedule. Three of 15 subjects (20 percent) on Schedule A, and 10 of 15 subjects (67 percent). Schedule B

---

[9] This definition of a vaccine responder is highly restrictive in light of the scientific objectives of this trial: e.g., to assess the feasibility of post-infection immunization.

developed an antibody increase to envelope proteins (P=0.025 by Fisher's exact test, two-tailed). All 13 subjects also seroconverted to specific envelope epitopes.

Conversely, of the 10 subjects who failed to seroconvert to any envelope specific epitope, none exhibited an increase in envelope antibody by Western Blot. The remaining 7 subjects who seroconverted to specific envelope epitopes demonstrated no change in whole virus envelope antibody by Western Blot. No changes in antibody directed against non envelope HIV proteins were observed in any subject.

Fourteen of 15 subjects (93 percent) on Schedule B (6 doses) demonstrated an increase in total gp160 antibody, as opposed to only 7 of 15 subjects (47 percent) on Schedule A (3 doses) (P=0.01 Fisher's, two-tailed). (Fig. 7).

As shown in Fig. 8, the pre-immunization to post-vaccination prevalence of each gp160 specific epitope respectively was as follows: Epitope 49 (27 to 70 percent), Epitope 88 (28 to 52 percent), Epitope 106 (50 to 87 percent), Epitope 214 (0 to 14 percent), Epitope 254 (0 to 13 percent), Epitope 300 (47 to 77 percent), Epitope 308 (42 to 69 percent), Epitope 342 (0 to 27 percent), Epitope 422 (3 to 10 percent), Epitope 448C (73 to 87 percent), and Epitope 735 (17 to 33 percent). Vaccine induced seroconversion was noted against all of the specific epitopes except 582 (Fig. 7). Antibodies (seroconversion) directed against Epitopes 241, 254 or 342 were only detected following vaccination.

Secondary immune responses were detected to the following epitopes: 88, 106, 300, 448C, and 582. The prevalence of antibody directed against epitope 582 was 100 percent pre-vaccination and only one subject (3 percent) demonstrated a secondary immune response.

The pattern of vaccine induced HIV antibody to envelope epitopes was variable (Fig. 7). Primary antibody responses (seroconversion) to at least one epitope occurred in 20 subjects; 14 of 15 receiving Schedule B, and 6 of 15

randomized to Schedule A (P=0.005 Fisher's, two-tailed). Schedule A subjects seroconverted to only 15 of 110 (14 percent) of the potential epitopes to which they had no preimmunization antibodies. Schedule B subjects seroconverted to 60 of 129 (47 percent) (P<0.0001 Fisher's, two-tailed). Seroconversion to three or more envelope epitopes occurred in 9 subjects (60 percent) randomized to Schedule B but only 2 subjects (13 percent) randomized to Schedule A (P=0.02 Fisher's, two-tailed).

Serum neutralization activity against three distinct strains (HIV-IIIB, MN, and RF) was determined on days 0, 90 and 195 in 7 subjects. Four of 5 vaccine responders demonstrated increasing neutralizing activity to one or more isolate. The vaccine responders also demonstrated an increased ability to inhibit syncytium formation compared to non-responders.

G. Vaccine Induced Cellular Responses

Changes in cellular immune response were based on a comparison of mean pre-vaccination (baseline) and post-vaccination lymphocyte stimulation indices (LSI) using a Wilcoxon rank sum test.

Twenty-one of 30 subjects (70 percent) developed a new T cell proliferative response to gp160 post-immunization (Fig. 7).

Figure 9 illustrates proliferative responses to gp160, p24 and a baculovirus control protein in four typical vaccine responders over time. For all subjects the gp160 induced proliferation increased from a baseline mean LSI of 3 to an LSI of 10 (calculated utilizing the mean of 4 values following the last immunization). In contrast, no change was noted for proliferative responses directed against HIV p24 protein or the control baculovirus protein.

Vaccine induced changes in mean LSI values for all subjects, for subjects subgrouped by vaccine responsiveness, and for subjects grouped by immunization schedule are illustrated in Figure 10.

The change in proliferative response to gp160 was significantly different between vaccine responders and non-responders (<0.001, Wilcoxon, one tailed). The gp160 proliferation responses induced by Schedule B (6 doses) were greater than those induced by Schedule A (3 doses) (P<0.10, Wilcoxon, one tailed).

Nineteen of the 21 subjects who developed proliferative responses to gp160 also developed a humoral response (vaccine responders). The maximum mean lymphocyte stimulation index (LSI) to gp160 observed for all vaccine responders was 50.1. However, each vaccine responder's response was variable (peak values ranging from a LSI of 3 to 171) (Fig. 7), as was the temporal relationship to vaccination of the magnitude and duration of the cellular responses to gp160 (Figure 9).

H. Discussion of Results

Despite the limited sample size of this trial, several factors were demonstrated to be associated with vaccine immunogenicity. Six of 15 (40 percent) of the subjects on Schedule A versus 13 of 15 (87 percent) of the subjects on Schedule B were vaccine responders (P=0.02 Fisher's, two-tailed) (Fig. 7). Of the 16 subjects with a mean baseline CD4 count greater than 600 per milliliter, 13 (81 percent) were vaccine responders, as opposed to 6 of 14 (43 percent) subjects whose mean entry CD4 count was less than 600 cells per milliliter (P=0.07 Fisher's, two-tailed). As summarized in Table 8, multiple immunizations improved immunogenicity, even among patients with baseline CD4 counts less than 600 cells per milliliter. For example, 5 of 6 subjects on Schedule B (6 injections) were vaccine responders as compared to only 1 of 8 who received the 3 injection regimen (Schedule A) P=0.03 Fisher's, two-tailed) (Table 8).

TABLE 8

GP 160 Vaccine Immune Responsiveness by Baseline CD4 Count and Immunization Schedule

CD4 Count	N	# Responders (%)	# Non Responders (%)
SCHEDULE A			
>600	7	5 (71%)	2 (29%)
500-600	5	1 (20%)	4 (80%)
<500	3	0 (0%)	3 (100%)
Subtotal	15	6 (40%)	9 (60%)
SCHEDULE B			
>600	9	8 (89%)	1 (11%)
500-600	2	2 (100%)	0 (0%)
<500	4	3 (75%)	1 (25%)
Subtotal	15	13 (87%)	2 (13%)
TOTAL	30	19 (63%)	11 (37%)

The therapeutic use of vaccines was introduced by Pasteur in the 19th century for the treatment of acute rabies infection. But the utility of this approach in the treatment of other infections has not been extensively explored. Although there are other examples of post infection modification of viral-specific immunity (such as after hepatitis A or B exposure), there are no well documented studies in man which demonstrate the feasibility of this approach for an established or chronic viral infection.

Here, the invention provides virus-specific immune modification by active immunization after infection. Specifically, an HIV envelope gene derived gp160 vaccine augmented the human host directed viral-specific humoral and cellular responses in 19 of 30 early HIV infected persons.

This study qualitatively and quantitatively measured distinct antibody responses to specific HIV epitopes in natural infection versus post infection immunization. In this way, an accurate determination of vaccine induced humoral immunogenicity in already infected persons was documented in 70 percent of the subjects. For example, twenty subjects (19 vaccine responders and 1 vaccine non-responder) seroconverted to specific envelope epitopes.

Seroconversion associated only with vaccination (epitopes 241, 254, and 342) occurred in 10 subjects.

Additionally, variations in humoral responses to this vaccine, as characterized by epitope mapping, will permit prospective cause and effect analysis of specific antibody responses, and allow unique opportunities to characterize potential immunoregulatory mechanisms not elicited during a natural infection.

Although the in vivo relevance of serum neutralizing activity is presently unknown, the observation of increased neutralizing activity against disparate HIV strains (IIIB, RF, MN) in 4 of 5 vaccine responders suggests that post-infection immunization induced changes in functional antibodies. The test vaccine induced increases in serum neutralization capacity against distinct HIV strains and will potentially aid in the definition of group specific neutralization epitopes.

A proliferative response to HIV envelope proteins rarely occurs in natural HIV infection. However, after immunization with gp160, specific T-cell proliferative respon-ses were documented in 21 (70 percent) of the subjects. The reason for this difference is unclear. One possibility is that the new proliferative response may be directed against an envelope epitope(s) unique to the vaccine (as a result of vaccine production methodology or alternative in vivo antigen processing). Alternatively, the protein used in the proliferation assay may not stimulate primary T-cell proliferative responses against homologous "wild type" envelopes of natural virus. However, additional evidence that vaccination boosts the host cellular immune response has been obtained: selected vaccine responders demonstrated HIV-IIIB type-specific cytotoxic T-cell responses following booster immunization.

The factors responsible for vaccine immunoresponsiveness in HIV infected persons remain to be clarified. Even in early HIV infection, individuals respond suboptimally to a variety of vaccines as compared to matched controls. This hyporesponsiveness has been related to early

B cell dysregulation and T-cell dysfunction. Here, vaccine immunoresponsiveness was associated with baseline CD4 cell count, which is consistent with the hypothesis that the immunological status of the host is an important determinant of vaccine responsiveness. However, the immunization schedule within specific T-cell count intervals also influenced vaccine responsiveness: Schedule B (6 injections) was superior. Indeed, the decreased vaccine response observed in the subjects with lower CD4 cell counts could be improved by an increased number of vaccinations which suggests that further modifications in dosage, regimen, adjuvants or formulation, could be anticipated to further improve host immunoresponsiveness.

Although concerns have been raised about the safety of active immunization of HIV infected persons with HIV specific vaccine products, there was no evidence of immune-specific toxicity. Quantitative cultures, DNA polymerase chain reaction assays and serum antigen assays show an increased in vivo HIV load An excellent in vivo marker of HIV replication, the rate of CD4 cell decline, was favorably influenced among the subjects, especially those classified as vaccine responders. The change in mean CD4 counts for responders was -0.2 percent and was -7.3 percent for non-responders. The data demonstrates that post-infection immune responsiveness was not associated with an increase in CD4 destruction and suggests an association with decreased HIV replication in vivo.

The vaccination results in this study were also compared with a database of ten infected and untreated individuals matched for age, ethnic group, and baseline CD4 cell count. The mean CD4 count decreased by 8.7 percent in this reference group, decreased by 7.2 percent in the subjects assigned to Schedule A, and increased by 0.6 percent in subjects assigned to Schedule B. These results indicate that post-infection vaccination with recombinant HIV envelope protein is feasible, and furthermore the result are encouraging with respect to the prophylactic uses of such vaccines.

WHAT IS CLAIMED IS:

1. A method for treating an individual infected with human immunodeficiency virus (HIV) comprising administering a recombinant HIV envelope protein to the infected individual.

2. A method according to claim 1, wherein the recombinant protein is administered in a dose of about 1 to 100 micrograms per kilogram of body weight.

3. A method according to claim 1, wherein the recombinant protein is administered in a dose of about 10μg to about 4000μg.

4. A method according to claim 1, wherein the recombinant protein is administered in a dose of about 40μg to about 1280μg.

5. A method according to claim 3, wherein at least three doses are administered.

6. A method according to claim 4, wherein at least six doses are administered.

7. A method according to claim 5, wherein each dose is administered at an interval of about 30 to 60 days.

8. A method according to claim 6, wherein each dose is administered at an interval of about 30 to 60 days.

9. A method for treating an individual infected with human immunodeficiency virus (HIV) comprising:

administering a recombinant HIV envelope protein to the infected individual in an amount sufficient to elicit an increase in HIV-specific cellular or humoral immune responses.

10. A method according to claim 1, wherein the recombinant protein is produced by a baculovirus insect cell expression system.

11. A method according to claim 3, wherein the recombinant protein is produced by a baculovirus insect cell expression system.

12. A method according to claim 5, wherein the recombinant protein is produced by a baculovirus insect cell expression system.

13. A method according to claim 1, wherein the recombinant protein has a molecular weight of approximately 145,000.

14. A method according to claim 3, wherein the recombinant protein has a molecular weight of approximately 145,000.

15. A method according to claim 5, wherein the recombinant protein has a molecular weight of approximately 145,000.

16. A method according to claim 1, wherein the HIV envelope protein is at least one of gp160, gp120, and gp41.

17. A method according to claim 3, wherein the HIV envelope protein is at least one of gp160, gp120, and gp41.

18. A method according to claim 5, wherein the HIV envelope protein is at least one of gp160, gp120, and gp41.

19. A method according to claim 1, wherein the recombinant protein is expressed by the baculovirus insect cell vector Ac3046.

20. A method according to claim 3, wherein the recombinant protein is expressed by the baculovirus insect cell vector Ac3046.

21. A method according to claim 5, wherein the recombinant protein is expressed by the baculovirus insect cell vector Ac3046.

22. A method according to claim 1, wherein the recombinant protein is agglomerated into particles having a molecular weight of at least about 2,000,000.

23. A method according to claim 3, wherein the recombinant protein is agglomerated into particles having a molecular weight of at least about 2,000,000.

24. A method according to claim 5, wherein the recombinant protein is agglomerated into particles having a molecular weight of at least about 2,000,000.

25. A method according to claim 1, wherein the recombinant protein is combined with an adjuvant.

26. A method according to claim 3, wherein the recombinant protein is combined with an adjuvant.

27. A method according to claim 5, wherein the recombinant protein is combined with an adjuvant.

28. A method for treating an individual infected with human immunodeficiency virus (HIV) comprising administering to an infected individual a composition including a recombinant HIV envelope protein and an alum adjuvant, wherein the recombinant protein is formed into particles having a molecular weight of at least about 2,000,000.

29. A method according to claim 28, wherein the recombinant protein is produced by a baculovirus insect cell expression system.

30. A method according to claim 28, wherein the recombinant protein is selected from the group consisting of recombinant gp160, recombinant gp120, recombinant gp41, a recombinant HIV envelope protein having a molecular weight of about 145,000, and a recombinant protein expressed by vector Ac3046.

31. A method according to claim 28, wherein the recombinant protein comprises about 757 successive amino acids of gp160 and substantially excludes about 40 successive terminal amino acids of gp160.

32. A method according to claim 28, wherein the recombinant protein is administered in a dose of about 10μg to about 4000μg.

33. A therapeutic HIV vaccine composition comprising a recombinant HIV envelope protein and an alum adjuvant, wherein the recombinant protein is formed into particles having a molecular weight of at least about 2,000,000.

34. A composition according to claim 33, wherein the recombinant HIV envelope protein is provided in an amount of about 10μg to 4000μg per dose.

35. A composition according to claim 34, wherein the recombinant protein is produced by a baculovirus insect cell expression system.

36. A composition according to claim 34, wherein the recombinant protein includes about 757 successive amino acids of gp160 and substantially excludes about 40 terminal amino acids of gp160.

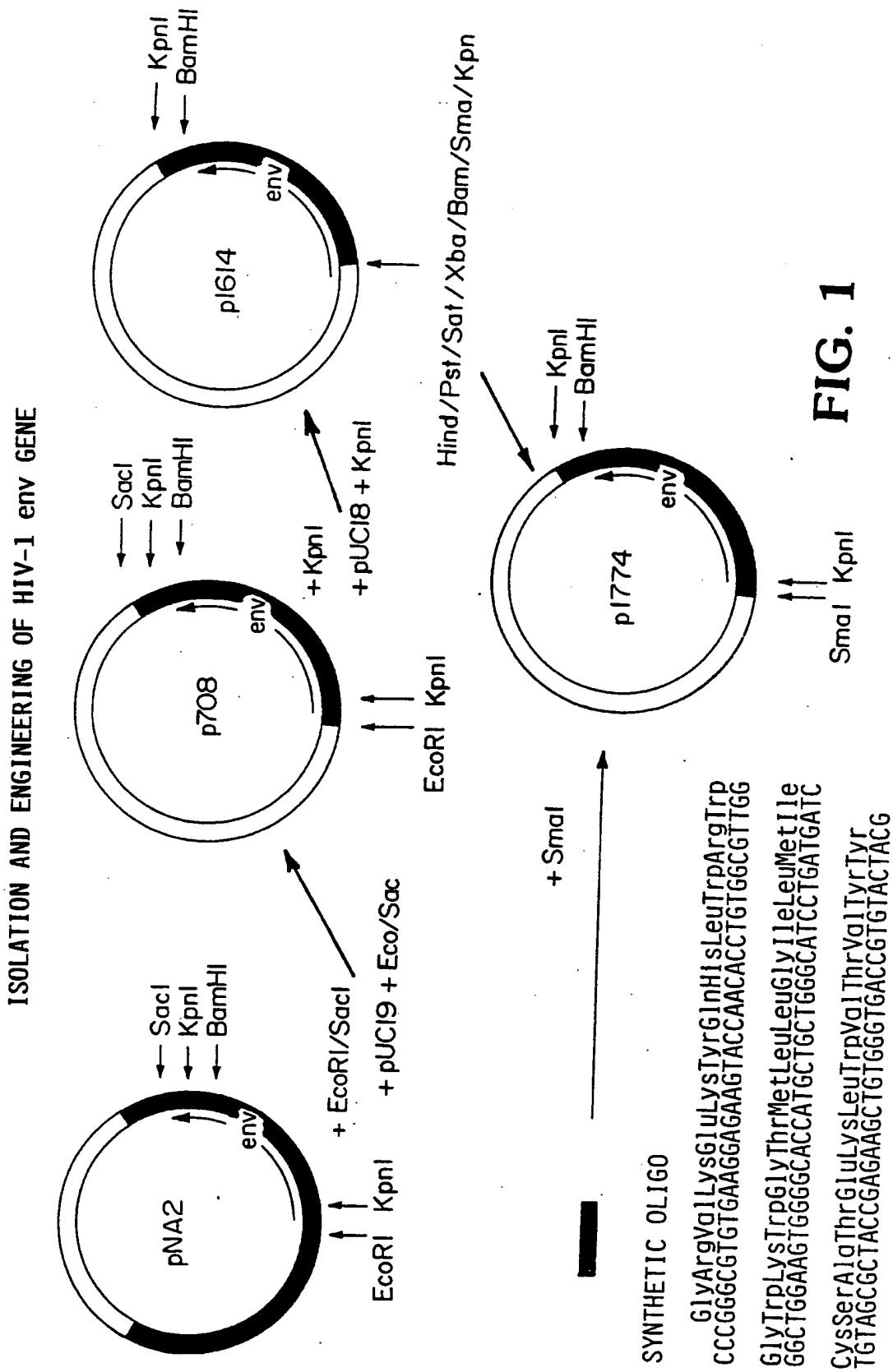

ISOLATION AND ENGINEERING OF HIV-1 env GENE
pNA2
env
SacI
KpnI
BamHI
EcoRI KpnI
+ EcoRI/SacI
+ pUC19 + Eco/Sac
p708
env
SacI
KpnI
BamHI
EcoRI KpnI
+ KpnI
+ pUC18 + KpnI
p1614
env
KpnI
BamHI
Hind/Pst/Sat/Xba/Bam/Sma/Kpn
+ SmaI
p1774
env
KpnI
BamHI
SmaI KpnI
SYNTHETIC OLIGO
GlyArgValLysGluLysTyrGlnHisLeuTrpArgTrp
CCCGGGCGTGTGAAGGAGAAGTACCAACACCTGTGGCGTTGG
GlyTrpLysTrpGlyThrMetLeuLeuGlyIleLeuMetIle
GGCTGGAAGTGGGGCACCATGCTGCTGGGCATCCTGATGATC
CysSerAlaThrGluLysLeuTrpValThrValTyrTyr
TGTAGCGCTACCGAGAAGCTGTGGGTGACCGTGTACTACG

FIG. 1

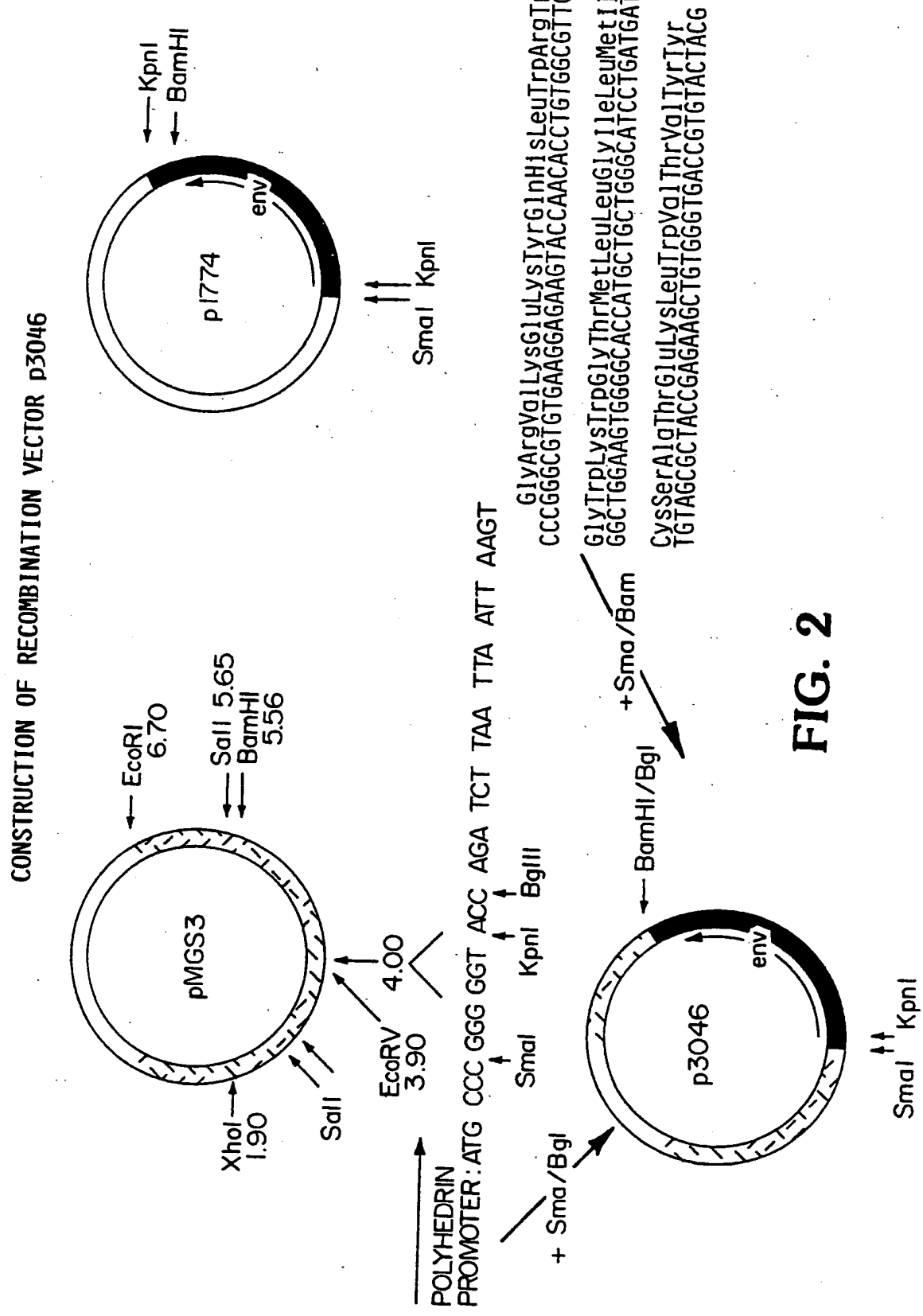

CONSTRUCTION OF RECOMBINATION VECTOR p3046
KpnI
BamHI
p1774
env
SmaI KpnI
EcoRI 6.70
SalI 5.65
BamHI 5.56
pMGS3
XhoI 1.90
SalI
EcoRV 3.90
4.00
POLYHEDRIN PROMOTER: ATG CCC GGG GGT ACC AGA TCT TAA TTA ATT AAGT
SmaI
KpnI
BglII
+ Sma/Bgl
BamHI/Bgl
+Sma/Bam
GlyArgValLysGluLysTyrGlnHisLeuTrpArgTrp
CCCGGGCGTGTGAAGGAGAAGTACCAACACCTGTGGCGTTGG
GlyTrpLysTrpGlyThrMetLeuLeuGlyIleLeuMetIle
GGCTGGAAGTGGGGCACCATGCTGCTGGGCATCCTGATGATC
CysSerAlaThrGluLysLeuTrpValThrValTyrTyr
TGTAGCGCTACCGAGAAGCTGTGGGTGACCGTGTACTACG
p3046
env
SmaI KpnI

FIG. 2

NUCLEOTIDE SEQUENCE OF DNA FLANKING
THE Ac3046 gp160 CODING SEQUENCES

TGCTGATATC ATGGAGATAA TTAAAATGAT AACCATCTCG CAAATAAATA
-100

AGTATTTTAC TGTTTTCGTA ACAGTTTTGT AATAAAAAAA CCTATAAATA
-50

ATG -----/3046/----- TAATTAATTAA GT ACC GAC TCT GCT GAA GAG
+1 +2257

GAG GAA ATT CTC CTT GAA GTT TCC CTG GTG TTC AAA GTA AAG GAG
+2287

TTT GCA CCA GAC GCA CCT CTG TTC ACT GGT CCG GCG TAT TAA
+2332 +2374

FIG. 3

NUCLEOTIDE SEQUENCE AND PREDICTED AMINO ACID SEQUENCE OF 3046 OPEN READING FRAME

FIG. 4a

With enzymes:

```
        SS
    AHN Ncc SS                  R
    vpc crr em                  s
    aai iFF ca                  a
    121 111 11                  1
     |  ||| ||
ATG CCC GGG CGT GTG AAG GAG AAG TAC CAA CAC CTG TGG CGT TGG
TAC GGG CCC GCA CAC TTC CTC TTC ATG GTT GTG GAC ACC GCA ACC
Met Pro Gly Arg Val Lys Glu Lys Tyr Gln His Leu Trp Arg Trp
                  5                  10                  15

                    B       F B                 SS      F
        B       B N sF  N   n s                 af      n
        b       a 1 po  1   u t                 ua      u
        v       n a 1k  a   4 X                 3N      4
        1       1 4 21  3   H 1                 A1      H
                     |                           |
GGC TGG AAG TGG GGC ACC ATG CTG CTG GGC ATC CTG ATG ATC TGC
CCG ACC TTC ACC CCG TGG TAC GAC GAC CCG TAG GAC TAC TAG ACG
Gly Trp Lys Trp Gly Thr Met Leu Leu Gly Ile Leu Met Ile Cys
                 20                  25                  30
```

Pst1	Hha1 Hae2		Bbv1	Alu1		BstE2	Mae3			Rsa1 Hph1		Ban1	Nla4 Rsa1	Kpn1
AGC	GCT	ACC	GAG	AAG	CTG	TGG	GTG	ACC	GTG	TAC	TAC	GGG	GTA	CCT
TCG	CGA	TGG	CTC	TTC	GAC	ACC	CAC	TGG	CAC	ATG	ATG	CCC	CAT	GGA
Ser	Ala	Thr	Glu	Lys	Leu	Trp	Val	Thr	Val	Tyr	Tyr	Gly	Val	Pro
				35					40					45

									SfaN1					SfaN1
GTG	TGG	AAG	GAA	GCA	ACC	ACC	ACT	CTA	TTT	TGT	GCA	TCA	GAT	GCT
CAC	ACC	TTC	CTT	CGT	TGG	TGG	TGA	GAT	AAA	ACA	CGT	AGT	CTA	CGA
Val	Trp	Lys	Glu	Ala	Thr	Thr	Thr	Leu	Phe	Cys	Ala	Ser	Asp	Ala
				50					55					60

FIG. 4b

FIG. 4c

	Nde1	Mn11			Tth32	Rsa1				Sau96	Hae3			N Ns 1p aH 31
AAA	GCA	TAT	GAT	ACA	GAG	GTA	CAT	AAT	GTT	TGG	GCC	ACA	CAT	GCC
TTT	CGT	ATA	CTA	TGT	CTC	CAT	GTA	TTA	CAA	ACC	CGG	TGT	GTA	CGG
Lys	Ala	Tyr	Asp	Thr	Glu	Val	His	Asn	Val	Trp	Ala	Thr	His	Ala
				65					70					75

	Rsa1													Mae3
TGT	GTA	CCC	ACA	GAC	CCC	AAC	CCA	CAA	GAA	GTA	GTA	TTG	GTA	AAT
ACA	CAT	GGG	TGT	CTG	GGG	TTG	GGT	GTT	CTT	CAT	CAT	AAC	CAT	TTA
Cys	Val	Pro	Thr	Asp	Pro	Asn	Pro	Gln	Glu	Val	Val	Leu	Val	Asn
				80					85					90

					Af13	Nla3	NspH1				S fN al Na 13			Mn11
GTG	ACA	GAA	AAT	TTT	AAC	ATG	TGG	AAA	AAT	GAC	ATG	GTA	GAA	CAG
CAC	TGT	CTT	TTA	AAA	TTG	TAC	ACC	TTT	TTA	CTG	TAC	CAT	CTT	GTC
Val	Thr	Glu	Asn	Phe	Asn	Met	Trp	Lys	Asn	Asp	Met	Val	Glu	Gln
				95					100					105

	Nsi1	Nla3						Sau3A			Bin1			
ATG	CAT	GAG	GAT	ATA	ATC	AGT	TTA	TGG	GAT	CAA	AGC	CTA	AAG	CCA
TAC	GTA	CTC	CTA	TAT	TAG	TCA	AAT	ACC	CTA	GTT	TCG	GAT	TTC	GGT
Met	His	Glu	Asp	Ile	Ile	Ser	Leu	Trp	Asp	Gln	Ser	Leu	Lys	Pro
				110					115					120

Nla3							Dra3			Dra1	ApaL1		BH sg pi 1A 21	
TGT	GTA	AAA	TTA	ACC	CCA	CTC	TGT	GTT	AGT	TTA	AAG	TGC	ACT	GAT
ACA	CAT	TTT	AAT	TGG	GGT	GAG	ACA	CAA	TCA	AAT	TTC	ACG	TGA	CTA
Cys	Val	Lys	Leu	Thr	Pro	Leu	Cys	Val	Ser	Leu	Lys	Cys	Thr	Asp
				125					130					135

				Mbo2
TTG	AAG	AAT	GAT	ACT
AAC	TTC	TTA	CTA	TGA
Leu	Lys	Asn	Asp	Thr
				140

FIG. 4d

FIG. 4e

AAT	ACC	AAT	AGT	AGT	AGC	GGG	AGA	ATG	ATA	ATG	GAG	AAA	GGA	GAG
TTA	TGG	TTA	TCA	TCA	TCG	CCC	TCT	TAC	TAT	TAC	CTC	TTT	CCT	CTC
Asn	Thr	Asn	Ser	Ser	Ser	Gly	Arg	Met	Ile	Met	Glu	Lys	Gly	Glu
				145					150					155

														Tth32
ATA	AAA	AAC	TGC	TCT	TTC	AAT	ATC	AGC	ACA	AGC	ATA	AGA	GAT	AAG
TAT	TTT	TTG	ACG	AGA	AAG	TTA	TAG	TCG	TGT	TCG	TAT	TCT	CTA	TTC
Ile	Lys	Asn	Cys	Ser	Phe	Asn	Ile	Ser	Thr	Ser	Ile	Arg	Asp	Lys
				160					165					170

					Bsm1 Nsi1								Rsa1	
GTG	CAG	AAA	GAA	TAT	GCA	TTC	TTT	TAT	AAA	CTT	GAT	ATA	GTA	CCA
CAC	GTC	TTT	CTT	ATA	CGT	AAG	AAA	ATA	TTT	GAA	CTA	TAT	CAT	GGT
Val	Gln	Lys	Glu	Tyr	Ala	Phe	Phe	Tyr	Lys	Leu	Asp	Ile	Val	Pro
				175					180					185

				Alu1						Mae3		Dde1		
ATA	GAT	AAT	ACC	AGC	TAT	AGG	TTG	ATA	AGT	TGT	AAC	ACC	TCA	GTC
TAT	CTA	TTA	TGG	TCG	ATA	TCC	AAC	TAT	TCA	ACA	TTG	TGG	AGT	CAG
Ile	Asp	Asn	Thr	Ser	Tyr	Arg	Leu	Ile	Ser	Cys	Asn	Thr	Ser	Val
				190					195					200

```
 M               HHS
 n               aat
 l               eeu
 1               131
                  ||
ATT  ACA  CAG  GCC  TGT  CCA  AAG  GTA  TCC  TTT  GAG  CCA  ATT  CCC  ATA
TTA  TGT  GTC  CGG  ACA  GGT  TTC  CAT  AGG  AAA  CTC  GGT  TAA  GGG  TAT
Ile  Thr  Gln  Ala  Cys  Pro  Lys  Val  Ser  Phe  Glu  Pro  Ile  Pro  Ile
                    205                      210                      215

                     B    SS                  H
                SA  sHN  Ncc   SS             i
                ev  ppc  crr   em             n
                ca  1ai  iFF   ca             f
                11  221  111   11             1
                    |||  |||   ||
CAT  TAT  TGT  GCC  CCG  GCT  GGT  TTT  GCG  ATT  CTA  AAA  TGT  AAT  AAT
GTA  ATA  ACA  CGG  GGC  CGA  CCA  AAA  CGC  TAA  GAT  TTT  ACA  TTA  TTA
His  Tyr  Cys  Ala  Pro  Ala  Gly  Phe  Ala  Ile  Leu  Lys  Cys  Asn  Asn
                    220                      225                      230

                               S
      M                        Aa        NR
      a                        vu        ls
      e                        a9        aa
      2                        26        31
                               |
AAG  ACG  TTC  AAT  GGA  ACA  GGA  CCA  TGT  ACA
TTC  TGC  AAG  TTA  CCT  TGT  CCT  GGT  ACA  TGT
Lys  Thr  Phe  Asn  Gly  Thr  Gly  Pro  Cys  Thr
                    235                      240
```

FIG. 4f

FIG. 4g

				Rsa1		Rsa1			Hinf1 Nla31		Hae1	Hae3		
AAT	GTC	AGC	ACA	GTA	CAA	TGT	ACA	CAT	GGA	ATC	AGG	CCA	GTA	GTA
TTA	CAG	TCG	TGT	CAT	GTT	ACA	TGT	GTA	CCT	TAG	TCC	GGT	CAT	CAT
Asn	Val	Ser	Thr	Val	Gln	Cys	Thr	His	Gly	Ile	Arg	Pro	Val	Val
				245					250					255

									Mae1					
TCA	ACT	CAA	CTG	CTG	TTA	AAT	GGC	AGT	CTA	GCA	GAA	GAA	GAT	GTA
AGT	TGA	GTT	GAC	GAC	AAT	TTA	CCG	TCA	GAT	CGT	CTT	CTT	CTA	CAT
Ser	Thr	Gln	Leu	Leu	Leu	Asn	Gly	Ser	Leu	Ala	Glu	Glu	Asp	Val
				260					265					270

Mbo2	Mbo2	Bgl2 Sau3A Xho2												
GTA	ATT	AGA	TCT	GCC	AAT	TTC	ACA	GAC	AAT	GCT	AAA	ACC	ATA	ATA
CAT	TAA	TCT	AGA	CGG	TTA	AAG	TGT	CTG	TTA	CGA	TTT	TGG	TAT	TAT
Val	Ile	Arg	Ser	Ala	Asn	Phe	Thr	Asp	Asn	Ala	Lys	Thr	Ile	Ile
				275					280					285

```
             N
R      A     sP                                                      R
s      l     pv                                                      s
a      u     Bu                                                      a
1      1     22                                                      1
             ||
GTA    CAG   CTG   AAC   ACA   TCT   GTA   GAA   ATT   AAT    TGT    ACA   AGA   CCC   AAC
CAT    GTC   GAC   TTG   TGT   AGA   CAT   CTT   TAA   TTA    ACA    TGT   TCT   GGG   TTG
Val    Gln   Leu   Asn   Thr   Ser   Val   Glu   Ile   Asn    Cys    Thr   Arg   Pro   Asn
                         290                           295                             300

                                                                           SBS
                                           M                         AFN   asc   S
                                           n                         vil   utr   e
                                           l                         ana   9NF   c
                                           1                         214   611   1
                                                                     ||    | |   |
AAC    AAT   ACA   AGA   AAA   AGT   ATC   CGT   ATC   CAG    AGG    GGA   CCA   GGG   AGA
TTG    TTA   TGT   TCT   TTT   TCA   TAG   GCA   TAG   GTC    TCC    CCT   GGT   CCC   TCT
Asn    Asn   Thr   Arg   Lys   Ser   Ile   Arg   Ile   Gln    Arg    Gly   Pro   Gly   Arg
                   305                                 310                             315

       M
       a
       e
       3
GCA    TTT   GTT   ACA   ATA
CGT    AAA   CAA   TGT   TAT
Ala    Phe   Val   Thr   Ile
                         320
```

FIG. 4h

FIG. 4i

										Mae3		Tth32		
GGA	AAA	ATA	GGA	AAT	ATG	AGA	CAA	GCA	CAT	TGT	AAC	ATT	AGT	AGA
CCT	TTT	TAT	CCT	TTA	TAC	TCT	GTT	CGT	GTA	ACA	TTG	TAA	TCA	TCT
Gly	Lys	Ile	Gly	Asn	Met	Arg	Gln	Ala	His	Cys	Asn	Ile	Ser	Arg
				325					330					335

				Bsm1		Dra1				Alu1 Mae1 Nhe1				
GCA	AAA	TGG	AAT	GCC	ACT	TTA	AAA	CAG	ATA	GCT	AGC	AAA	TTA	AGA
CGT	TTT	ACC	TTA	CGG	TGA	AAT	TTT	GTC	TAT	CGA	TCG	TTT	AAT	TCT
Ala	Lys	Trp	Asn	Ala	Thr	Leu	Lys	Gln	Ile	Ala	Ser	Lys	Leu	Arg
				340					345					350

												Mnl1	Dde1	Mst2
GAA	CAA	TTT	GGA	AAT	AAT	AAA	ACA	ATA	ATC	TTT	AAG	CAA	TCC	TCA
CTT	GTT	AAA	CCT	TTA	TTA	TTT	TGT	TAT	TAG	AAA	TTC	GTT	AGG	AGT
Glu	Gln	Phe	Gly	Asn	Asn	Lys	Thr	Ile	Ile	Phe	Lys	Gln	Ser	Ser
				355					360					365

```
                           P    S
          A    DFM   NNp   a          M
          v    rin   llu   u          a                                 M
          a    anl   aaM   9          e                                 n
          2    211   441   6          3                                 l
               |||   |||   |                                            1
GGA  GGG  GAC  CCA  GAA   ATT  GTA  ACG  CAC  AGT   TTT  AAT  TGT  GGA  GGG
CCT  CCC  CTG  GGT  CTT   TAA  CAT  TGC  GTG  TCA   AAA  TTA  ACA  CCT  CCC
Gly  Gly  Asp  Pro  Glu   Ile  Val  Thr  His  Ser   Phe  Asn  Cys  Gly  Gly
                    370                       375                       380

                                                               R    S
                                                               s    c
                                                               a    a
                                                               1    1
                                                               |    |
GAA  TTT  TTC  TAC  TGT   AAT  TCA  ACA  CAA  CTG   TTT  AAT  AGT  ACT  TGG
CTT  AAA  AAG  ATG  ACA   TTA  AGT  TGT  GTT  GAC   AAA  TTA  TCA  TGA  ACC
Glu  Phe  Phe  Tyr  Cys   Asn  Ser  Thr  Gln  Leu   Phe  Asn  Ser  Thr  Trp
                    385                       390                       395

          R    S
          s    c
          a    a
          1    1
               |
TTT  AAT  AGT  ACT  TGG
AAA  TTA  TCA  TGA  ACC
Phe  Asn  Ser  Thr  Trp
                    400
```

FIG. 4j

FIG. 4k

Rsa1	Sca1							Eco57		Mae3				Eco57
AGT	ACT	GAA	GGG	TCA	AAT	AAC	ACT	GAA	GGA	AGT	GAC	ACA	ATC	ACA
TCA	TGA	CTT	CCC	AGT	TTA	TTG	TGA	CTT	CCT	TCA	CTG	TGT	TAG	TGT
Ser	Thr	Glu	Gly	Ser	Asn	Asn	Thr	Glu	Gly	Ser	Asp	Thr	Ile	Thr
				405					410					415

		Nla3							Af13	Nla3	NspH1			
CTC	CCA	TGC	AGA	ATA	AAA	CAA	TTT	ATA	AAC	ATG	TGG	CAG	GAA	GTA
GAG	GGT	ACG	TCT	TAT	TTT	GTT	AAA	TAT	TTG	TAC	ACC	GTC	CTT	CAT
Leu	Pro	Cys	Arg	Ile	Lys	Gln	Phe	Ile	Asn	Met	Trp	Gln	Glu	Val
				420					425					430

									Mn11					
GGA	AAA	GCA	ATG	TAT	GCC	CCT	CCC	ATC	AGT	GGA	CAA	ATT	AGA	TGT
CCT	TTT	CGT	TAC	ATA	CGG	GGA	GGG	TAG	TCA	CCT	GTT	TAA	TCT	ACA
Gly	Lys	Ala	Met	Tyr	Ala	Pro	Pro	Ile	Ser	Gly	Gln	Ile	Arg	Cys
				435					440					445

	Bbv1	Ssp1				Fnu4H								
TCA	TCA	AAT	ATT	ACT	GGG	CTG	CTA	TTA	ACA	AGA	GAT	GGT	GGT	AAT
AGT	AGT	TTA	TAA	TGA	CCC	GAC	GAT	AAT	TGT	TCT	CTA	CCA	CCA	TTA
Ser	Ser	Asn	Ile	Thr	Gly	Leu	Leu	Leu	Thr	Arg	Asp	Gly	Gly	Asn
				450					455					460

	Eco57		Ava2 / Mbo2	Nla4 / Sau96	Bgl2 / Sau3A	Xho2	Mn11	Mn11	BstNI / ScrF1	Gsu1		Mn11		
AAC	AAC	AAT	GGG	TCC	GAG	ATC	TTC	AGA	CCT	GGA	GGA	GGC	GAT	ATG
TTG	TTG	TTA	CCC	AGG	CTC	TAG	AAG	TCT	GGA	CCT	CCT	CCG	CTA	TAC
Asn	Asn	Asn	Gly	Ser	Glu	Ile	Phe	Arg	Pro	Gly	Gly	Gly	Asp	Met
				465					470					475

Fin1				
AGG	GAC	AAT	TGG	AGA
TCC	CTG	TTA	ACC	TCT
Arg	Asp	Asn	Trp	Arg
				480

FIG. 41

FIG. 4m

```
AGT GAA TTA TAT AAA  TAT AAA GTA GTA AAA  ATT GAA CCA TTA GGA
TCA CTT AAT ATA TTT  ATA TTT CAT CAT TTT  TAA CTT GGT AAT CCT
Ser Glu Leu Tyr Lys  Tyr Lys Val Val Lys  Ile Glu Pro Leu Gly
                485                   490                  495

                SS                             M
                et                             b
                cy                             o
                11                             2
                 |
GTA GCA CCC ACC AAG  GCA AAG AGA AGA GTG  GTG CAG AGA GAA AAA
CAT CGT GGG TGG TTC  CGT TTC TCT TCT CAC  CAC GTC TCT CTT TTT
Val Ala Pro Thr Lys  Ala Lys Arg Arg Val  Val Gln Arg Glu Lys
                500                   505                  510

                                                            F
                          A        S   S                    n
                          l        e   t                    u
                          u        c   y                    4
                          1        1   1                    H
                                       |
AGA GCA GTG GGA ATA  GGA GCT TTG TTC CTT  GGG TTC TTG GGA GCA
TCT CGT CAC CCT TAT  CCT CGA AAC AAG GAA  CCC AAG AAC CCT CGT
Arg Ala Val Gly Ile  Gly Ala Leu Phe Leu  Gly Phe Leu Gly Ala
                515                   520                  525
```

			Bbv1 Hga1			Fnu4H Hha1				Bbv1		Hga1	Rsa1	
GCA	GGA	AGC	ACT	ATG	GGC	GCA	GCG	TCA	ATG	ACG	CTG	ACG	GTA	CAG
CGT	CCT	TCG	TGA	TAC	CCG	CGT	CGC	AGT	TAC	TGC	GAC	TGC	CAT	GTC
Ala	Gly	Ser	Thr	Met	Gly	Ala	Ala	Ser	Met	Thr	Leu	Thr	Val	Gln
				530					535					540

Hae1 Hae3									Fnu4H	Fnu4H		Bbv1	Mnl1 Bbv1	
GCC	AGA	CAA	TTA	TTG	TCT	GAT	ATA	GTG	CAG	CAG	CAG	AAC	AAT	TTG
CGG	TCT	GTT	AAT	AAC	AGA	CTA	TAT	CAC	GTC	GTC	GTC	TTG	TTA	AAC
Ala	Arg	Gln	Leu	Leu	Ser	Asp	Ile	Val	Gln	Gln	Gln	Asn	Asn	Leu
				545					550					555

Dde1				
CTG	AGG	GCT	ATT	GAG
GAC	TCC	CGA	TAA	CTC
Leu	Arg	Ala	Ile	Glu
				560

FIG. 4n

FIG. 40

					S f a N 1									S f a N 1
GCG	CAA	CAG	CAT	CTG	TTG	CAA	TCT	ACA	GTC	TGG	GGC	ATC	AAA	CAA
CGC	GTT	GTC	GTA	GAC	AAC	GTT	AGA	TGT	CAG	ACC	CCG	TAG	TTT	GTT
Ala	Gln	Gln	His	Leu	Leu	Gln	Ser	Thr	Val	Trp	Gly	Ile	Lys	Gln
				565					570					575

G s u 1	BS sc tr NF 11		TH ti hn 3f 21		BS sc tr NF 11							S a u 3 A		
CTC	CAG	GCA	AGA	ATC	CTG	GCT	GTG	GAA	AGA	TAC	CTA	AAG	GAT	CAA
GAG	GTC	CGT	TCT	TAG	GAC	CGA	CAC	CTT	TCT	ATG	GAT	TTC	CTA	GTT
Leu	Gln	Ala	Arg	Ile	Leu	Ala	Val	Glu	Arg	Tyr	Leu	Lys	Asp	Gln
				580					585					590

BA il nu 11	S e c 1	BS sc tr NF 11												
CAG	CTC	CTG	GGG	ATT	TGG	GGT	TGC	TCT	GGA	AAA	CTC	ATT	TGC	ACC
GTC	GAG	GAC	CCC	TAA	ACC	CCA	ACG	AGA	CCT	TTT	GAG	TAA	ACG	TGG
Gln	Leu	Leu	Gly	Ile	Trp	Gly	Cys	Ser	Gly	Lys	Leu	Ile	Cys	Thr
				595						600				605

```
     M         SS                  MB
     m         et                  as
     e         cy                  em
     1         11                  11
               |
ACT GCT GTG CCT TGG AAT GCT AGT TGG AGT AAT AAA TCT CTG GAA
TGA CGA CAC GGA ACC TTA CGA TCA ACC TCA TTA TTT AGA GAC CTT
Thr Ala Val Pro Trp Asn Ala Ser Trp Ser Asn Lys Ser Leu Glu
                610                 615                 620

                                B   S
                        N       s   c           F   F
                        l       t   r           i   o
                        a       N   F           n   k
                        3       1   1           1   1
                                |   |
CAG ATT TGG AAT AAC ATG ACC TGG ATG GAG TGG GAC AGA GAA AAT
GTC TAA ACC TTA TTG TAC TGG ACC TAC CTC ACC CTG TCT CTT TAA
Gln Ile Trp Asn Asn Met Thr Trp Met Glu Trp Asp Arg Glu Ile
                625                 630                 635

             H
             i   A
             n   l
             d   u
             3   1
AAC AAT TAC ACA AGC TTA
TTG TTA ATG TGT TCG AAT
Asn Asn Tyr Thr Ser Leu
                640
```

FIG. 4p

FIG. 4q

```
                              H        M
                              i        b
                              n        o
                              f        2
                              1
ATA CAC TCC TTA ATT GAA GAA TCG CAA AAC CAG CAA GAA AAG AAT
TAT GTG AGG AAT TAA CTT CTT AGC GTT TTG GTC GTT CTT TTC TTA
Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn
            645                 650                 655

GAA CAA GAA TTA TTG GAA TTA GAT AAA TGG GCA AGT TTG TGG AAT
CTT GTT CTT AAT AAC CTT ATT CTA TTT ACC CGT TCA AAC ACC TTA
Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn
            660                 665                 670

TGG TTT AAC ATA ACA AAT TGG CTG TGG TAT ATA AAA TTA TTC ATA
ACC AAA TTG TAT TGT TTA AAC GAC ACC ATA TAT TTT AAT AAG TAT
Trp Phe Asn Ile Thr Asn Trp Leu Trp Tyr Ile Lys Leu Phe Ile
            675                 680                 685

 M                                                          R
 n                                                          s
 l                                                          a
 1                                                          1
ATG ATA GTA GGA GGC TTG GTA GGT TTA AGA ATA GTT TTT GCT GTA
TAC TAT CAT CCT CCG AAC CAT CCA AAT TCT TAT CAA AAA CGA CAT
Met Ile Val Gly Gly Leu Val Gly Leu Arg Ile Val Phe Ala Val
            690                 695                 700
```

```
                                                H
                                                p
                                                h
                                                1
CTT TCT ATA GTG AAT AGA GTT AGG CAG GGA TAT TCA CCA TTA TCG
GAA AGA TAT CAC TTA TCT CAA TCC GTC CCT ATA AGT GGT AAT AGC
Leu Ser Ile Val Asn Arg Val Arg Gln Gly Tyr Ser Pro Leu Ser
            705                 710                 715

                                                 P   S   S
                    M   A   MS      ADF NNp  a  aH
                    n   v   ne      vri 11u  u  ua
                    1   a   1c      aan aaM  9  9e
                    1   1   11      221 441  6  63
                            |        || |||  |
TTT CAG AAC CAC CTC CCA ATC CCG AGG GGA CCC GAC AGG CCC GAA
AAA GTC TGG GTG GAG GGT TAG GGC TCC CCT GGG CTG TCC GGG CTT
Phe Gln Thr His Leu Pro Ile Pro Arg Gly Pro Asp Arg Pro Glu
            720                 725                 730

                        M   M
                        b   b
                        o   o
                        2   2
GGA ATA GAA GAA GAA GGT GGA GAG AGA GAC
CCT TAT CTT CTT CTT CCA CCT CTC TCT CTG
Gly Ile Glu Glu Glu Gly Gly Glu Arg Asp
            735                 740
```

FIG. 4r

FIG. 4S

```
 B           S              T                                              S                   B
 i           aX             a                                              aX                  i
 n           uh             q                                              uh                  n
 1           3o             1                                              3o                  1
             A2                                                            A2
             |                                                             |
AGA   GAC   AGA   TCC   ATT   CGA   TTA   GTG   AAC   GGA   TCT   TAA   TTA   ATT   AA
TCT   CTG   TCT   AGG   TAA   GCT   AAT   CAC   TTG   CCT   AGA   ATT   AAT   TAA   TT
Arg   Asp   Arg   Ser   Ile   Arg   Leu   Val   Asn   Gly   Ser   End   Leu   Ile
                  745                           750                     755
```

Enzymes that do cut:

Afl3	Alu1	ApaL1	Ava1	Ava2	Ban1	Bbv1	Bgl2	Bin1	Bsm1	Bsp12
BstE2	BstN1	BstX1	Dde1	Dra1	Dra2	Dra3	Eco57	Fin1	Fnu4H	Fok1
Gsu1	Hae1	Hae2	Hae3	Hga1	HgiA1	Hha1	Hind3	Hinf1	Hpa2	Hph1
Kpn1	Mae1	Mae2	Mae3	Mbo2	Mme1	Mnl1	Mst2	Nci1	Nde1	Nhe1
Nla3	Nla4	Nsi1	NspB2	NspH1	PpuM1	Pst1	Pvu2	Rsa1	Sau3A	Sau96
Sca1	Scrf1	Sec1	SfaN1	Sma1	Ssp1	Stu1	Sty1	Taq1	Tth32	Xho2

Enzymes that do not cut:

Aat2	Acc1	Afl2	Aah2	Apa1	Asu2	Avr2	Bal1	BamH1	Ban2	Bbv2
Bcl1	Bgl1	BspH1	BspM1	BspM2	BssH2	Cfr1	Cfr10	Cla1	Dsa1	Eco31
EcoB	EcoK	EcoR1	EcoRV	Esp1	Fsp1	Gdi2	HgiE2	Hinc2	Hpa1	Mlu1
Nae1	Nar1	Nco1	Not1	Nru1	PflM1	PMaC1	Pvu1	Rsr2	Sac1	Sac2
Sal1	Sfi1	SnaB1	Spe1	Sph1	Spl1	Tha1	Tth31	Xba1	Xho1	Xma3
Xmn1										

Fig. 4t

NUMBER OF OPEN READING FRAME BASES: 2253

NUMBER OF AMINO ACID CODONS: = 2253 + 3 = 751

AMINO ACID	NUMBER	WEIGHT.	TOTALS
GLY -	53	75.1	3,980.3
GLU -	41	147.1	6,031.1
ASP -	25	133.1	3,327.5
VAL -	48	117.1	5,620.8
ALA -	37	89.1	3,296.7
ARG -	39	174.1	6,793.8
SER -	28	105.1	2,942.8
LYS -	42	146.2	6,140.4
ASN -	58	132.1	7,661.8
MET -	17	149.2	2,536.4
ILE -	57	131.2	7,478.4
THR -	53	119.1	6,312.3
TRP -	26	204.2	5,309.2
CYS -	21	121.2	2,545.2
TYR -	16	181.2	2,899.2
LEU -	61	131.2	8,003.2
PHE -	25	165.2	4,130.0
SER -	26	105.1	2,732.6
GLN -	38	146.2	5,555.6
HIS -	11	155.2	1,707.2
PRO -	29	115.1	3,337.9
TOTALS	751		98,342.4
			$-H_2O$ (751 x 18)

Total estimated weight of non-glycosylated polypeptide = 84,824.4

Total number of glycosylation sites:
28 x 2100 (wt per oligo saccharide)

Total estimated mol. wt. of gp160 = 84,824.4 + 58800

= 143,624.

Fig. 5a

COMPARISON OF LAV-1 AND RECOMBINANT Ac3046 pg160* SEQUENCE

The sequence and corresponding codons on the top lines are those predicted from the engineering and by Wain-Hobson et al. (1985). The sequence along the bottom of each line is that which was determined for Ac3046 from recombinant viral DNA.

Fig. 5b

Fig. 5c

```
                                                              190  ¥
Ala  Phe  Phe  Tyr  Lys  Leu  Asp  Ile  Ile  Pro  Ile  Asp  Asn  Asp  Thr
GCA  TTT  TTT  TAT  AAA  CTT  GAT  ATA  ATA  CCA  ATA  GAT  AAT  GAT  ACT     6802
|||  ||C  |||  |||  |||  |||  |||  |||  G||  |||  |||  |||  |||  (--------)
     Phe                                Val

                                        ¥
Thr  Ser  Tyr  Thr  Leu  Thr  Ser  Cys  Asn  Thr  Ser  Val  Ile  Thr  Gln
ACC  AGC  TAT  ACG  TTG  ACA  AGT  TGT  AAC  ACC  TCA  GTC  ATT  ACA  CAG     6847
|||  |||  |||  |G|  |||  |T|  |||  |||  |||  |||  |||  |||  |||  |||  |||
               Arg       Ile

     210
Ala  Cys  Pro  Lys  Val  Ser  Phe  Glu  Pro  Ile  Pro  Ile  His  Tyr  Cys
GCC  TGT  CCA  AAG  GTA  TCC  TTT  GAG  CCA  ATT  CCC  ATA  CAT  TAT  TGT     6892
     |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||

                              230                      ¥
Ala  Pro  Ala  Gly  Phe  Ala  Ile  Leu  Lys  Cys  Asn  Asn  Lys  Thr  Phe
GCC  CCG  GCT  GGT  TTT  GCG  ATT  CTA  AAA  TGT  AAT  AAT  AAG  ACG  TTC     6937
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||

¥                                  ¥                   250
Asn  Gly  Thr  Gly  Pro  Cys  Thr  Asn  Val  Ser  Thr  Val  Gln  Cys  Thr
AAT  GGA  ACA  GGA  CCA  TGT  ACA  AAT  GTC  AGC  ACA  GTA  CAA  TGT  ACA     6982
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||

                                                                 ¥
His  Gly  Ile  Arg  Pro  Val  Val  Ser  Thr  Gln  Leu  Leu  Leu  Asn  Gly
CAT  GGA  ATT  AGG  CCA  GTA  GTA  TCA  ACT  CAA  CTG  CTG  TTG  AAT  GGC     7027
|||  |||  ||C  |||  |||  |||  |||  |||  |||  |||  |||  |||  ||A  |||  |||
          Ile                                              Leu
```

```
     270                                                                 ¥
Ser  Leu  Ala  Glu  Glu  Glu  Val  Val  Ile  Arg  Ser  Ala  Asn  Phe  Thr
AGT  CTA  GCA  GAA  GAA  GAG  GTA  GTA  ATT  AGA  TCT  GCC  AAT  TTC  ACA
|||  |||  |||  |||  |||  ||T  |||  |||  |||  |||  |||  |||  |||  |||  |||   7072
                         Asp

                              290                 ¥
Asp  Asn  Ala  Lys  Thr  Ile  Ile  Val  Gln  Leu  Asn  Gln  Ser  Val  Glu
GAC  AAT  GCT  AAA  ACC  ATA  ATA  GTA  CAG  CTG  AAC  CAA  TCT  GTA  GAA
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  AC|  |||  |||  |||   7117
                                                       Thr

     ¥                                  ¥                   310
Ile  Asn  Cys  Thr  Arg  Pro  Asn  Asn  Asn  Thr  Arg  Lys  Ser  Ile  Arg
ATT  AAT  TGT  ACA  AGA  CCC  AAC  AAC  AAT  ACA  AGA  AAA  AGT  ATC  CGT
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||   7162

Ile  Gln  Arg  Gly  Pro  Gly  Arg  Ala  Phe  Val  Thr  Ile  Gly  Lys  Ile
ATC  CAG  AGG  GGA  CCA  GGG  AGA  GCA  TTT  GTT  ACA  ATA  GGA  AAA  ATA
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||   7207

     330                                     ¥
Gly  Asn  Met  Arg  Gln  Ala  His  Cys  Asn  Ile  Ser  Arg  Ala  Lys  Trp
GGA  AAT  ATG  AGA  CAA  GCA  CAT  TGT  AAC  ATT  AGT  AGA  GCA  AAA  TGG
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||   7252

¥                             350
Asn  Ala  Thr  Leu  Lys  Gln  Ile  Ala  Ser  Lys  Leu  Arg  Glu  Gln  Phe
AAT  GCC  ACT  TTA  AAA  CAG  ATA  GCT  AGC  AAA  TTA  AGA  GAA  CAA  TTT
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||   7297

          ¥                                                 370
Gly  Asn  Asn  Lys  Thr  Ile  Ile  Phe  Lys  Gln  Ser  Ser  Gly  Gly  Asp
GGA  AAT  AAT  AAA  ACA  ATA  ATC  TTT  AAG  CAA  TCC  TCA  GGA  GGG  GAC
|||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||  |||   7342
```

Fig. 5d

Fig. 5e

7387 7432 7477 7522 7567 7612 7657

Asp GAT Met ATG Arg AGG Asp GAC Asn AAT Trp TGG Arg AGA Ser AGT Glu GAA Leu TTA Tyr TAT Lys AAA Val GTA 7702
Val GTA Lys AAA Ile ATT Glu GAA Pro CCA Leu TTA Gly GGA Val GTA Ala GCA Pro CCC Thr ACC Lys AAG Ala GCA Lys AAG Arg AGA 7747
510 gp120
Arg AGA Val GTG Val GTG Gln CAG Arg AGA Glu GAA Lys AAA Arg AGA Ala GCA Val GTG Gly GGA Ile ATA Met ATG Gly GGC Ala GCA Ala GCT Arg CGG 7792
gion Phe TTC Leu CTT Gly GGG Phe TTC Leu TTG Gly GGA Ala GCA Ala GCA Gly GGA Ser AGC Thr ACT Met ATG Gly GGC Ala GCA Ala GCT Arg GC| Ala 7837
Ser TCA Met ATG Thr ACG Leu CTG Thr ACG Val GTA Gln CAG Ala GCC Arg AGA Gln CAA Leu TTA Leu TTG Ser TCT Gly GGT |A| Asp Ile ATA 7882
Val GTG Gln CAG Gln CAG Gln CAG Asn AAC Asn AAT Leu TTG Leu CTG Arg AGG Ala GCT Ile ATT Glu GAG Ala GCG Gln CAA Gln CAG 7927
His CAT Leu CTG Leu TTG Leu CTC Gln CAA Leu CTC Thr ACA Val GTC Trp TGG Gly GGC Ile ATC Lys AAG |A Gln CAG Leu CTC Gln CAG Ala GCA 7972

Fig. 5f

Fig. 5g

```
                              590
Arg Ile Leu Ala Val Glu Arg Tyr Leu Lys Asp Gln Gln Leu Leu
AGA ATC CTG GCT GTG GAA AGA TAC CTA AAG GAT CAA CAG CTC CTG
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||   8017

                                            610
Gly Ile Trp Gly Cys Ser Gly Lys Leu Ile Cys Thr Thr Ala Val
GGG ATT TGG GGT TGC TCT GGA AAA CTC ATT TGC ACC ACT GCT GTG
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||   8062

         ¥                   ¥
Pro Trp Asn Ala Ser Trp Ser Asn Lys Ser Leu Glu Gln Ile Trp
CCT TGG AAT GCT AGT TGG AGT AAT AAA TCT CTG GAA CAG ATT TGG
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||   8107

     ¥                                              ¥
Asn Asn Met Thr Trp Met Glu Trp Asp Arg Glu Ile Asn Asn Tyr
AAT AAC ATG ACC TGG ATG GAG TGG GAC AGA GAA ATT AAC AAT TAC
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||   8152

                        650
Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln
ACA AGC TTA ATA CAT TCC TTA ATT GAA GAA TCG CAA AAC CAG CAA
||| ||| ||| ||| ||C ||| ||| ||| ||| ||| ||| ||| ||| ||| |||   8197
                His

                                            670
Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser
GAA AAG AAT GAA CAA GAA TTA TTG GAA TTA GAT AAA TGG GCA AGT
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||   8242

                     ¥
Leu Trp Asn Trp Phe Asn Ile Thr Asn Trp Leu Trp Tyr Ile Lys
TTG TGG AAT TGG TTT AAC ATA ACA AAT TGG CTG TGG TAT ATA AAA
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||   8287
```

690
Ile ATA T Leu
Phe TTC
Ile ATA
Met ATG
Ile ATA
Val GTA
Gly GGC
Leu TTG
Val GTA
Gly GGT
Leu TTA
Arg AGA
Ile ATA
Val GTT
8332
Phe TTT
Ala GCT
Val GTA
Leu CTT
Ser TCT
Ile ATA
710
Val GTG
Asn AAT
Arg AGA
Val GTT
Arg AGG
Gln CAG
Gly GGA
Tyr TAT
Ser TCA
8377
Pro CCA
Leu TTA
Ser TCG
Phe TTT
Gln CAG
Thr ACC
His CAC
Leu CTC
Pro CCA
Thr ACC T Ile
Pro CCG
730
Arg AGG
Gly GGA
Pro CCC
Asp GAC
8422
Arg AGG
Pro CCC
Glu GAA
Gly GGA
Ile ATA
Glu GAA
Glu GAA
Gly GGT
Gly GGA
Glu GAG
Arg AGA
Asp GAC
Arg AGA
Asp GAC
8467
Arg AGA
8470
750
Ser TCC
Ile ATT
Arg CGA
Leu TTA
Val GTG
Asn AAC
Gly GGA
Ser TCT
END TAA
TTA
ATT
AA

Fig. 5h

FIG. 6

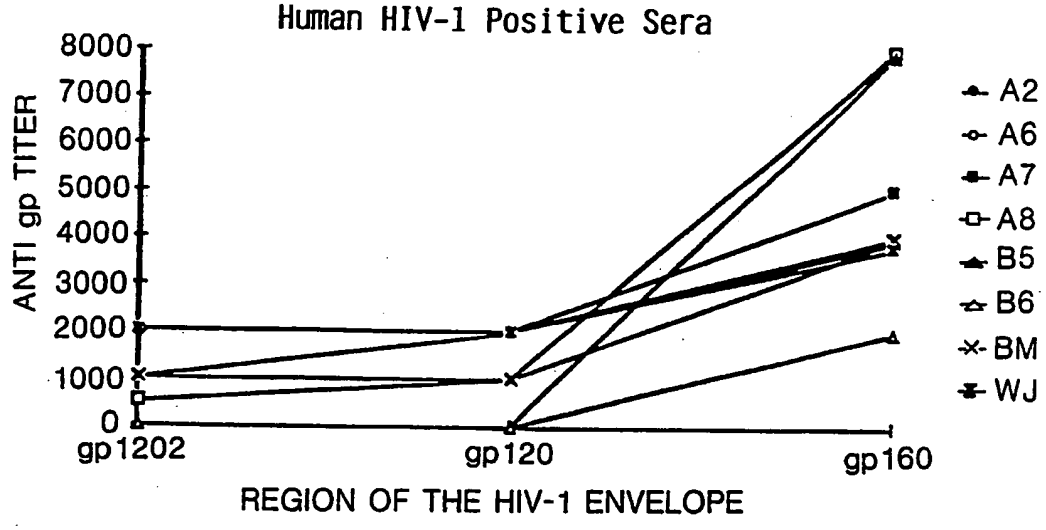
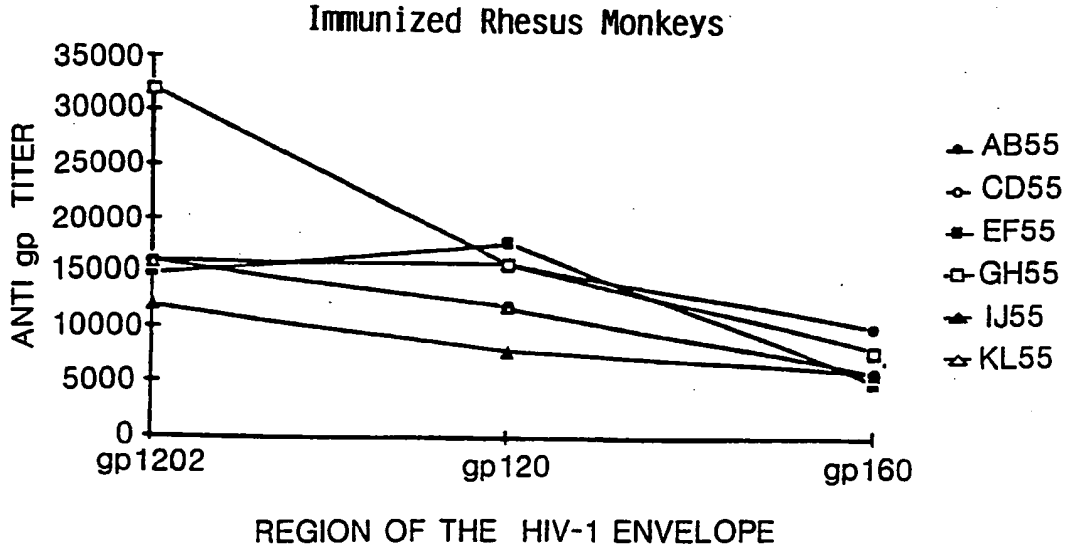

Human HIV-1 Positive Sera
ANTI gp TITER
8000
7000
6000
5000
4000
3000
2000
1000
0
gp1202
gp120
gp160
REGION OF THE HIV-1 ENVELOPE
A2
A6
A7
A8
B5
B6
BM
WJ

FIG. 6a


Immunized Rhesus Monkeys
ANTI gp TITER
35000
30000
25000
20000
15000
10000
5000
0
gp1202
gp120
gp160
REGION OF THE HIV-1 ENVELOPE
AB55
CD55
EF55
GH55
IJ55
KL55

Summary gp 160 Vaccine Induced Immune Responses

FIG. 7a

	Patient	Group	Humoral Antibody Response to Specific Epitopes												Cellular Response T-cell Proliferation
RESPONDERS			49	88	106	241	254	300	308	342	422	448	582	735	Total gp 160 (peak I.SI)
	8	1	+	+	+	-	-	[illegible]	[illegible]	[illegible]	+	[illegible]	+	+	[illegible] (25)
	15	1	[illegible]	+	+	-	[illegible]	+	+	-	-	+	+	=	[illegible] (71)
Schedule	17	1	[illegible]	-	+	-	-	+	+	-	-	+	+	+	[illegible] (23)
A	10	5	[illegible]	[illegible]	[illegible]	[illegible]	-	[illegible]	[illegible]	-	-	[illegible]	+	-	[illegible] (50)
	20	5	-	hb	[illegible]	-	-	-	-	-	-	-	+	-	[illegible] (19)
	22	5	+	-	+	-	-	-	-	-	-	+	+	-	[illegible] (16)
	1	2	[illegible]	[illegible]	[illegible]	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	+	-	[illegible] (102)
	3	2	[illegible]	-	[illegible]	-	-	-	-	-	-	+	+	[illegible]	[illegible] (171)
	16	2	[illegible]	[illegible]	-	-	-	[illegible]	-	-	-	+	+	-	[illegible] (41)
	19	2	[illegible]	[illegible]	[illegible]	-	-	[illegible]	[illegible]	[illegible]	-	+	+	-	[illegible] (20)
	6	4	[illegible]	[illegible]	[illegible]	-	-	-	-	-	-	+	+	-	[illegible] (34)
Schedule	7	4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	-	[illegible]	+	[illegible]	[illegible] (95)
B	13	4	+	+	+	-	-	+	+	-	-	+	+	[illegible]	[illegible] (27)
	28	4	-	[illegible]	+	-	-	[illegible]	nd	-	-	-	+	-	[illegible] (3)
	32	4	[illegible]	[illegible]	[illegible]	-	[illegible]	[illegible]	[illegible]	[illegible]	-	+	+	[illegible]	[illegible] (35)
	11	6	+	[illegible]	[illegible]	-	-	[illegible]	[illegible]	-	-	+	+	+	[illegible] (24)
	23	6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	+	[illegible]	[illegible] (8)
	29	6	[illegible]	-	[illegible]	hb	-	[illegible]	hb	[illegible]	-	+	+	-	[illegible] (158)
	33	6	[illegible]	-	[illegible]	-	-	[illegible]	+	[illegible]	-	+	+	-	[illegible] (28)

FIG. 7b

	Patient	Group	Humoral Antibody Response to Specific Epitopes										Cellular Response T-cell Proliferation
			49	88	106	241	254	300	308	342	422	448	582	735	Total gp 160 (peak I.SI)
NON-RESPONDERS	2	1	-	-	+	-	-	+	+	-	-	+	+	-	-
	18	1	-	+	-	-	-	-	-	-	-	-	+	-	-
	5	3	+	-	-	-	-	+	+	-	-	+	+	+	-
Schedule A	12	3	+	+	+	-	-	-	-	-	-	+	+	+	-
	14	3	+	-	+	-	-	+	+	-	-	+	+	-	-
	21	3	-	-	+	hb	-	+	hb	-	-	+	+	-	-
	35	3	-	-	▒	-	-	-	-	-	+	+	+	-	-
	27	5	-	-	+	-	-	+	nd	-	-	-	+	-	▒ (10)
	31	5	+	-	-	-	-	+	+	-	-	+	+	-	-
Schedule B	4	2	-	-	▒	-	-	+	+	-	-	+	+	-	-
	24	6	-	-	+	-	-	+	+	-	-	-	+	-	▒ (28)

FIG. 8a

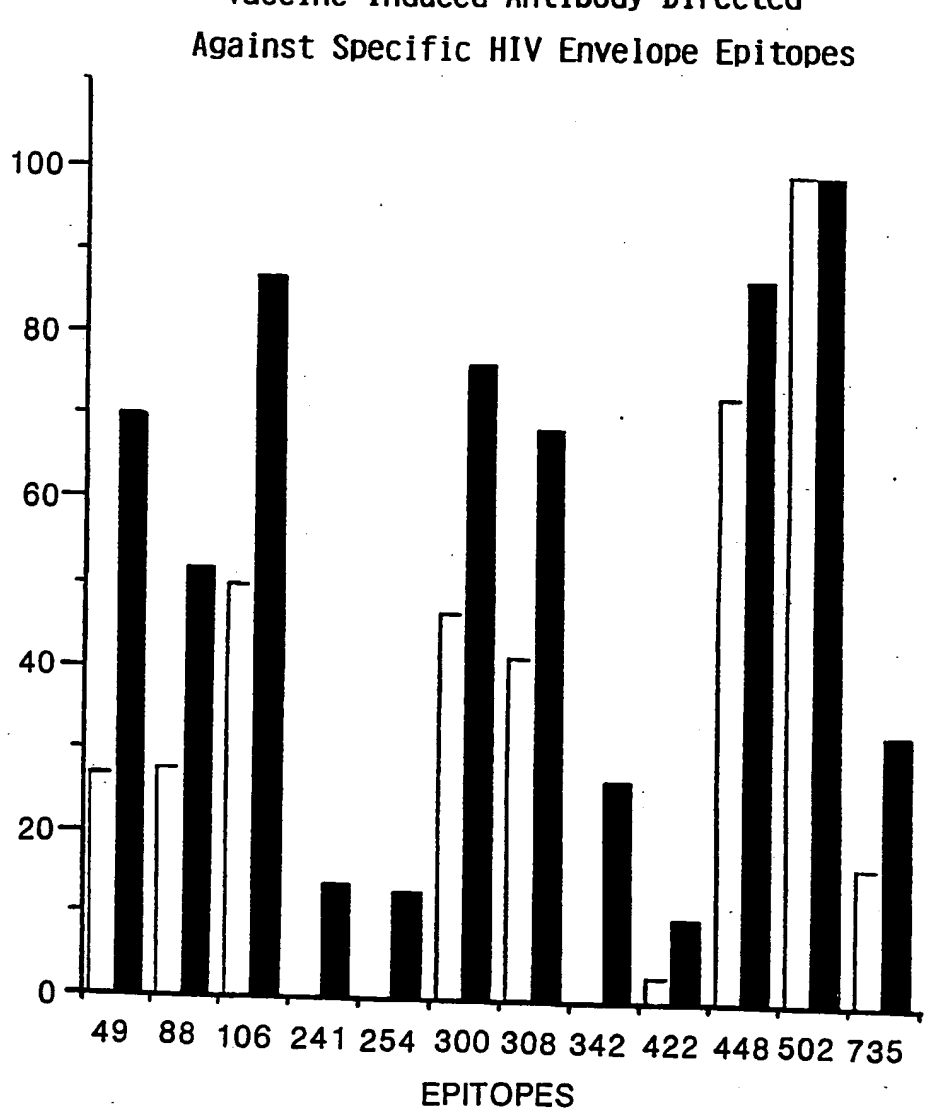

Vaccine Induced Antibody Directed
Against Specific HIV Envelope Epitopes
100
80
60
40
20
0
49 88 106 241 254 300 308 342 422 448 502 735
EPITOPES

FIG. 8b

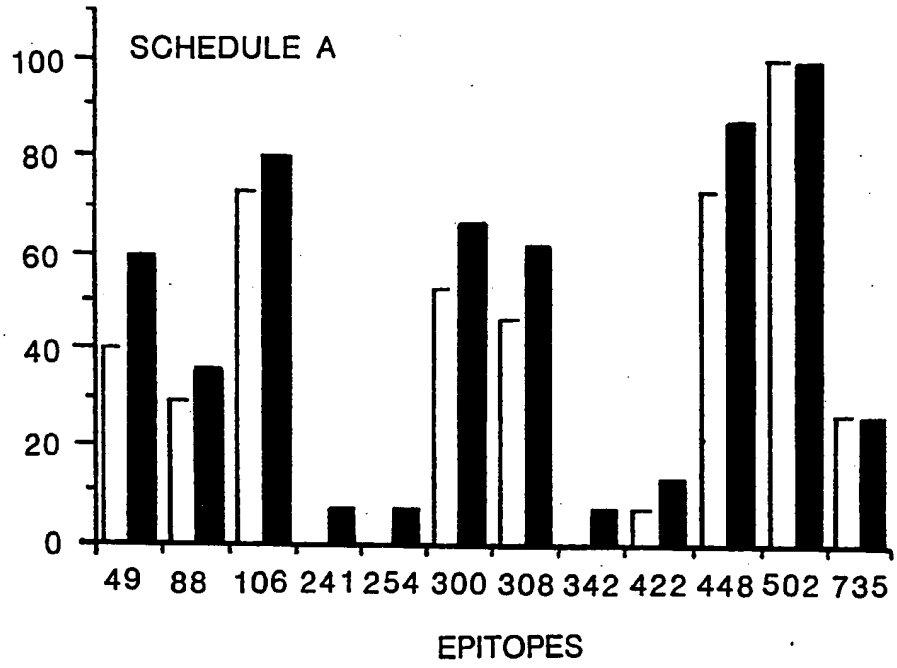
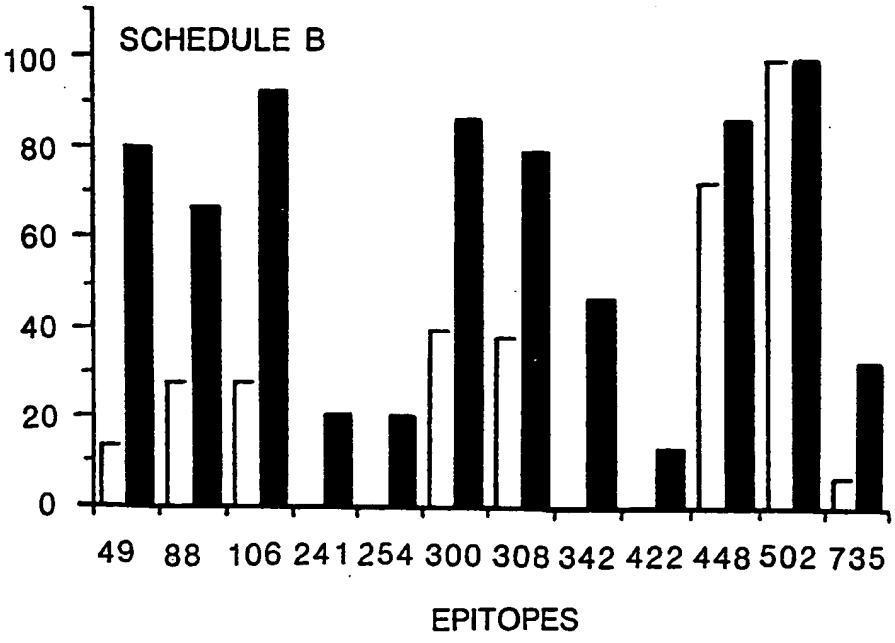
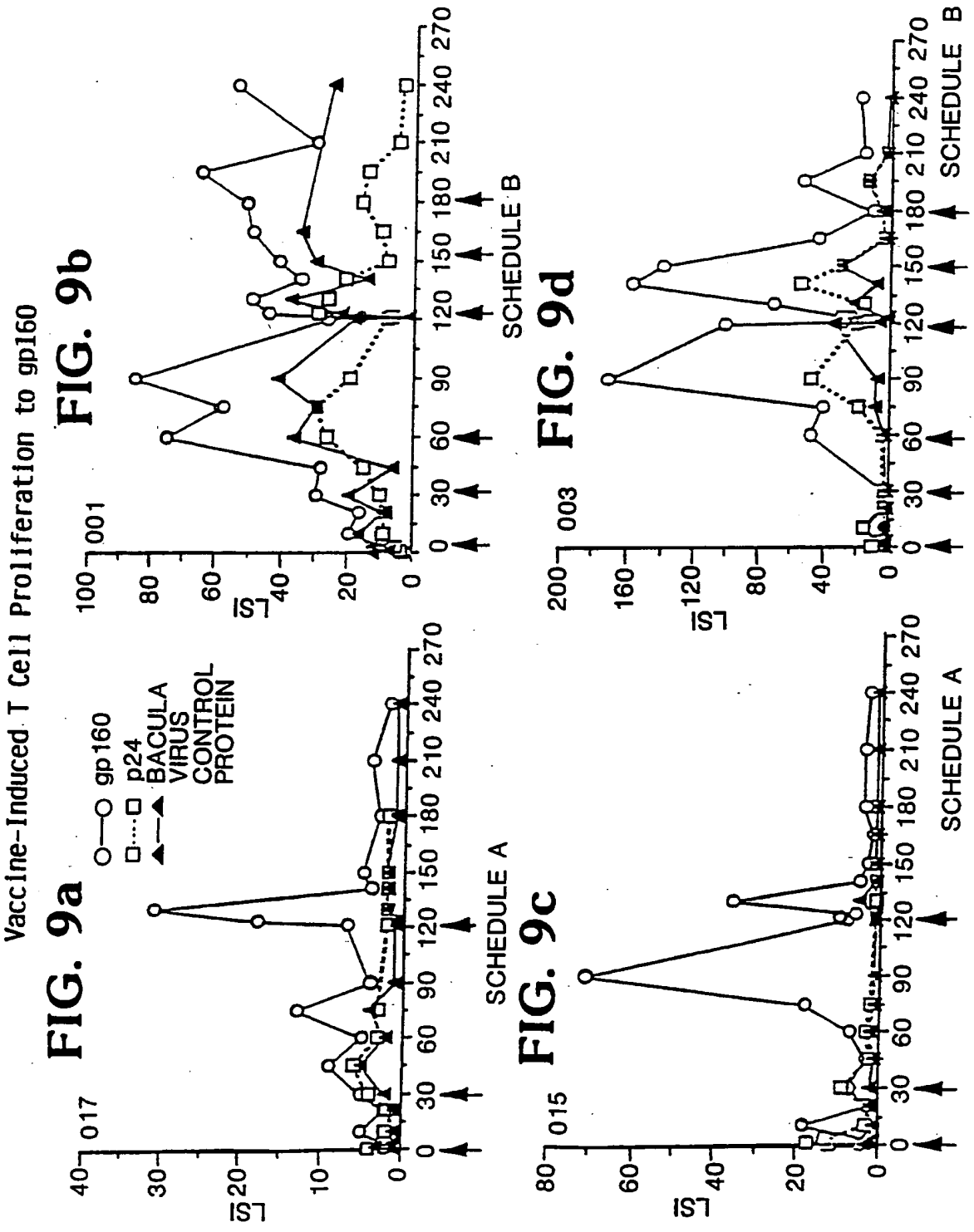

SCHEDULE A
100
80
60
40
20
0
49 88 106 241 254 300 308 342 422 448 502 735
EPITOPES

FIG. 8c


SCHEDULE B
100
80
60
40
20
0
49 88 106 241 254 300 308 342 422 448 502 735
EPITOPES


Vaccine-Induced T Cell Proliferation to gp160
FIG. 9a
FIG. 9b
FIG. 9c
FIG. 9d
gp160
p24
BACULA VIRUS CONTROL PROTEIN
SCHEDULE A
SCHEDULE B
LSI
017
001
015
003
0 30 60 90 120 150 180 210 240 270

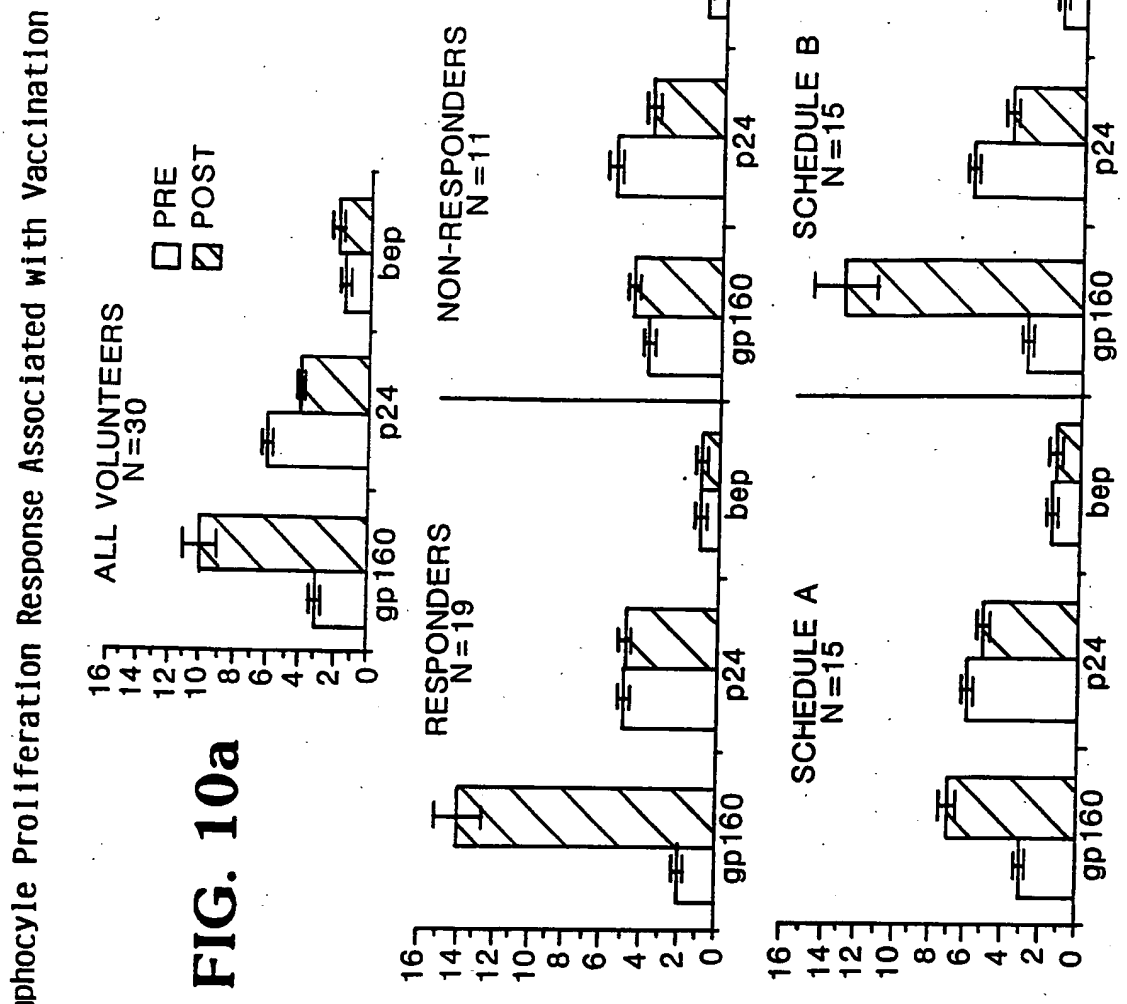
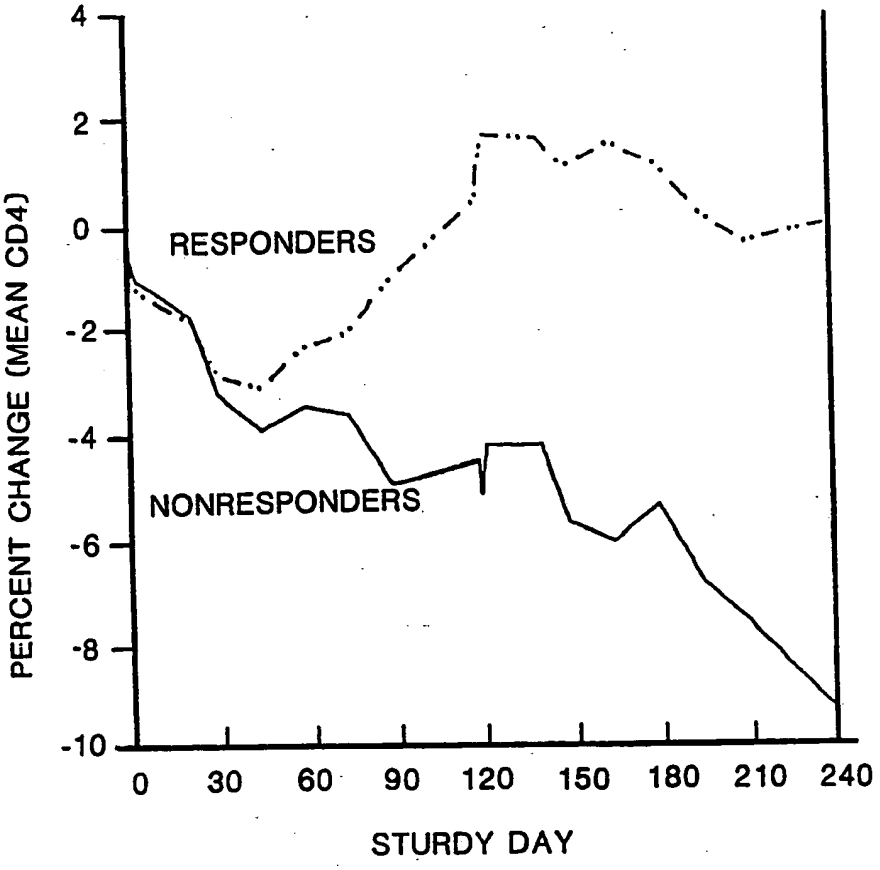


FIG. 10a

FIG. 10b

FIG. 10c

FIG. 11


4
2
0
-2
-4
-6
-8
-10
PERCENT CHANGE (MEAN CD4)
RESPONDERS
NONRESPONDERS
0
30
60
90
120
150
180
210
240
STURDY DAY

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/04980

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/49; C12N15/86; A61K39/21; C07K13/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 327 180 (MICROGENESYS INC.) 9 August 1989 see the whole document	1-36

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 SEPTEMBER 1992	19. 10. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

Form PCT/ISA/210 (second sheet) (January 1985)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04980

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1 to 32 are directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9204980 SA 61379

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0327180	09-08-89	AU-A- 2955789	03-08-89
		JP-A- 2203793	13-08-90

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.